The Developmental Genetics of Mouse

Teratocarcinoma and Embryonal Cells

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Abstract

Methods for the fusion of cell lines and the subsequent isolation of hybrid lines have been modified to enable the isolation of hybrids from two embryonal carcinoma (ec) cell lines.These two cell lines differed from each other in that one,PSA4,was capable of gap-junction mediated cell-cell communication (metabolic cooperation),whereas the other line,R5/3OA,was deficient in metabolic cooperation (mec-).Cells of the line PSA4 undergo spontaneous differentiation both *in vitro* in the absence of STO feeder cells and on subcutaneous injection into isogenic mice.R5/3OA cells on the other hand do not have these properties.

The two cell lines R5/3OA and PSA4 were fused in monolayer culture using PolyEthylene Glycol 1000 dissolved in HEPES buffer and were cultured initially in "enriched medium" which was modified for the purpose.Hybrid lines were selected in medium which contained Hypoxanthine,Aminopterin and Thymidine (HAT) and Ouabain,using the assumption that the Ouabain resistance of the R5/3OA parent line and the ability to grow in HAT medium displayed by the PSA4 parent,would both be present in the hybrid line.

Several putative hybrid lines were characterised initially by karyotyping and identified by the presence of 2 metacentric marker chromosomes,derived from R5/3OA and a sub-hexaploid chromosome count.It was found that the ability of gap- junction mediated communication and the ability to differentiate spontaneously were both dominant or semi-dominant characteristics and that the hybrids isolated displayed both of these features.

One hybrid line PR3 was then chosen as a basis for the isolation of segregant lines to enable a more rigorous investigation of the relationship between differentiation and metabolic cooperation.PR3Tg12,a thioguanine- resistant (HAT sensitive) variant of PR3,was used as the starting point for the selection of communication-defective segregants by the "Kiss of Death" procedure,i.e coculture with wild-type cells (in this case PR3) in the presence of thioguanine.PR3 cells make the enzyme Hypoxanthine-Guanine Phosphoribosyl transferase (HGPRT) which is lethal to the cells in the presence of thioguanine,Cooperation positive cells without this gene (PR3Tg12) were therefore also vulnerable to thioguanine in the presence of PR3 cells which pass on enzyme products via gap-junctional transfer.Two lines selected in this

way (Kd1a and Kd11Ba) were further characterised and one line (Kd1a) and its clonal derivative (Kd1a.6) were found to be deficient in metabolic cooperation.Analysis of the differentiative properties of these lines using *in vitro* (embryoid body formation) and *in vivo* (tumour formation) methods has shown that all of these lines differentiate as well in embryoid bodies as did the parent line (Tg12) but that *in vivo* this was not the case and only the original hybrid lines (PR3,PR5) formed differentiated tumours while PR3TG12,Kd1a.6 and Kd11Ba formed tumours which were predominantly of ec cell type.

A method for isolating cell lines with reduced differentiation ability is described and of six lines analysed by *in vitro* embryoid body formation,four had substantially reduced differentiation.Three of these lines were cooperation positive while the other had reduced cooperation ability. The isolation of Mec- cell lines still able to differentiate and lines with reduced potency which are mec+ indicates that the metabolic co-operation deficiency of R5/3OA is not causally related to its reduced developmental capacity.The different results obtained *in vivo* and *in vitro* suggests that the two situations are directed by different factors but this requires further investigation by a more rigorous analysis of the tumour derived cells.

Chapter 7 reports the isolation and characterisation of a cell line (Mod1) derived from a C57Bl.Mod1^{null} mouse blastocyst.This feeder dependent line was shown by electron and light microscopy to have a non-ec cell like morphology.Immunological and biochemical assays have confirmed these observations.Mod1 cells resemble parietal endoderm and produce large amounts of periodic acid Schiff (PAS) positive extracellular matrix.Mod1 has a normal diploid mouse karyotype and undergoes morphological changes when introduced to a non-feeder environment.

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AFP - Alpha-fetoprotein
APRT - Adenine phosphoribosyl transferase
BSA - Bovine Serum Albumin
CM - Complete Medium (see appendix 1)
CM10 - CM supplemented with 10% calf serum (app.1)
CM20 - CM supplemented with 20% calf serum (app.1)
CMX - Serumless Complete Medium
dCK - deoxyCytidine kinase
DDW - Deionised Distilled Water
DMSO - Dimethyl Sulphoxide
DNA - Deoxyribose nucleic acid
EB - Embryoid body
EC - Embryonal Carcinoma
EC10 - Embryoid body culture medium,10% calf serum (app.1)
EC20 - Embryoid body culture medium,20% calf serum (app.1)
EDTA - Ethylene diamine tetraacetic acid
EGTA - Ethylene glycol-bis-(B-aminoethylether)-N,N,N',N',-tetraacetic acid
EK - ec-like cell line isolated from a blastocyst
EM - Electron microscope
esc - embryonal stem cell (synonomous with EK)
FCS - Foetal calf serum
Gln - Glutamine (amino acid)
GMEM - Glasgow's modification of Eagles medium (app.1)
GPS - Guinea pig serum (complement)
G6PD - Glucose 6-phosphate dehydrogenase
GVBD - Germinal vesicle break-down
HAT - Hypoxanthine,Aminopterin,Thymidine
HCG - Human chorionic gonadotrophin
H&E - Haemotoxylin and Eosin
HFL - Human fibroblast line
HnRNA- Heterogeneous nuclear RNA
HPRT (HGPRT) - Hypoxanthine-(Guanine) phosphoribosyl transferase
HRP - Horse radish peroxidase
HSD - $^{5}\Delta$-3B Hydroxysteroid dehydrogenase
ICM - Inner cell mass
IF - Intermediate filament
i.u. - International Units
LD50 - Lethal dose (50% kill)
LM - Light microscope
MPB - Medium plastic bottle (75 cm^2)
mRNA - Messenger RNA
MW - Molecular Weight
NAD - B-Nicotinamide adenine dinucleotide
NADP - B-Nicotinamide adenine dinucleotide phosphate
NBT - Nitroblue Tetrazolium
NEAA - Non-essential amino acid
NCS - Newborn calf serum
OP - Orotate phosphoribosyl transferase
Oua - Ouabain
PAS - Periodic Acid Schiff's
PBS - Phosphate buffered saline (complete)
PBSA -phosphate buffered saline Ca^{2+} & Mg^{2+} free (app.1)
PE - Parietal Endoderm

PEG 1000 - Polyethylene Glycol (MW 1000)
PEG 6000 - Polyethylene Glycol (MW 6000)
PMS - Phenazine Methosulphate
PMSG - Pregnant mare serum (Gonadotrophin)
PrE - Primitive Endoderm
PVP - Polyvinyl Pyrrolidone
Pyr - Pyruvate
RER - Rough endoplasmic reticulum
RNA - Ribose nucleic acid
SPB - small plastic bottle (25 cm^2)
TCA - Trichloroacetic acid
TE - Trophectoderm
6-Tg - 6-Thioguanine
TK - Thymidine kinase
tRNA - Transfer RNA
TVP - Trypsin,EDTA,Chicken serum in PBSA
VE - Visceral Endoderm
VEE - Embryonic visceral endoderm
VEX - Extrembryonic visceral endoderm
ZP - Zona pellucida

CHAPTER 1

INTRODUCTION

1.1. General Introduction

The title of this work,"The Developmental Genetics of Mouse Embryonal and Teratocarcinoma Cells"reflects the aim of this project which is concerned with the possible relationship between normal early development in the mouse and the formation of tumours,and in particular with those tumours which can be shown to be manipulated by their environment,such as embryonal carcinomas (also known variously as teratocarcinomas and teratomas [1.4]),and with how such environmental effects interact with the genotype of the organism and to look at those areas where there are any demonstrable genotypic or epigenetic causes for these changes.

Therefore,with the aid of somatic cell genetic techniques,and with the culturing of normal embryos *in vitro*,it is hoped that we can find out something of the interaction between environmental and genetic influences and their effect on the normal developing embryo and on the seemingly uncontrolled growth patterns observed during tumour formation and cancer cell proliferation.

It is becoming increasingly apparent that there must be several different and possibly conflicting processes involved in the pathogenesis of the wide variety of disorders that come under the collective title of "Cancerous Diseases",and this is reflected by the wide spectrum of current research which,however tenuously,comes under the heading of "Cancer Research".Some of the relevant aspects of this research are discussed in this chapter.

It may not,therefore be entirely coincidental that Cancer Biology and Early Development remain two of the most notable areas of biological and medical understanding which are the least understood,and it may be that the elucidation of the one could lead to great advances in the other.

As stated by Evans and Kauffman [38],there is an intimate relationship between cancer and differentiation,which has been demonstrated experimentally by the many studies which have shown the interchangability of properties between malignant and embryonic cells.It is these properties and the cells which

manifest them which are the subject of this study.

For this reason it has therefore been necessary to consider in detail the early embryonic development of the mouse,with particular reference to the preimplantation stages during which occur the first cell differentiations concurrently with rapid cell division and increase in cell number.The most plausible mechanisms for these differentiations involve some kind of cell-cell interaction and so the literature which reports examples of such interactions is reviewed and one communication system in particular,that of Metabolic Cooperation and its relationship,if any,to differentiation,is discussed in detail as being the major system investigated in this thesis.

The majority of work in this project was done using embryonal carcinoma cells (ec cells) and so a review of the early history of this experimental system is included,as is also current work and a critique of the validity,advantages and disadvantages of the use of such cell lines as a model for development.

1.2. Development of the Mouse Embryo to Implantation

The mechanisms whereby information is passed from one generation to the next are carefully controlled both genetically and environmentally,and present both conceptual and practical difficulties to the understanding of these processes.Conceptual problems tend to arise from the difficulty of resolving the scientific approach to problem solving,which necessitates the isolation of certain topics within a subject and attempts to elucidate their function to give a linear understanding of events.This conflicts with the essentially circular process of development and its control.How do cells order both the totipotency of the germ line cells and the "nullipotency",or reduced potency , of more differentiated cells using the same genetic information and what role does the microenvironment play?

Therefore in my discussion of mouse development,I shall start and finish with the germ cell.Following the gradual increase in complexity of the organism and the reduction in potency of the majority of its component cells,until eventually there results a new organism complete with its own germ cells and capable of repeating the whole process again.

The development of the mouse embryo from gamete stage to implantation

stage is summarised in Figure 1:1 [1,72].A mouse embryo begins life with the fusion of two haploid gametes,one male and one female,in the proces known as fertilisation.From here the embryo goes through a well documented series of stages which are described firstly by cell number,as there is a rapid increase in cell numbers during the cleavage period,then latterly (as cells differentiate into various cell types) the stages are described by embryo appearance and by the major events taking place at the time.

The late morula goes into the blastocyst stage with the formation of the blastocoel.This is followed by the first differentiation of cells into embryonic and extraembryonic tissues with the formation of trophoblast and inner cell mass cells.The egg cylinder stage then appears as some cells become more differentiated forming endoderm and ectoderm tissues.

Simultaneously with these events,the whole egg moves down from the ampulla where it was fertilised round the loops of the oviduct until,by the end of the morula stage it has reached the uterus.Shortly after this the eggs space out along the length of the uterine horn,and implantation takes place,at the blastocyst stage.The egg cylinder is the first stage after the eggs have implanted and at this stage the extraembryonic structure of the placenta,yolk sac and amnion establish themselves.The foetus itself then continues to develop and more of its component cells become differentiated into special tissues by the processes of mesoderm formation,organogenesis and gastrulation.With the formation of the germ cells at 14-72 days the developmental cycle is nearing completion,although the mouse is not born until the 18th-19th days.

1.2.1. Oogenesis and Ovulation

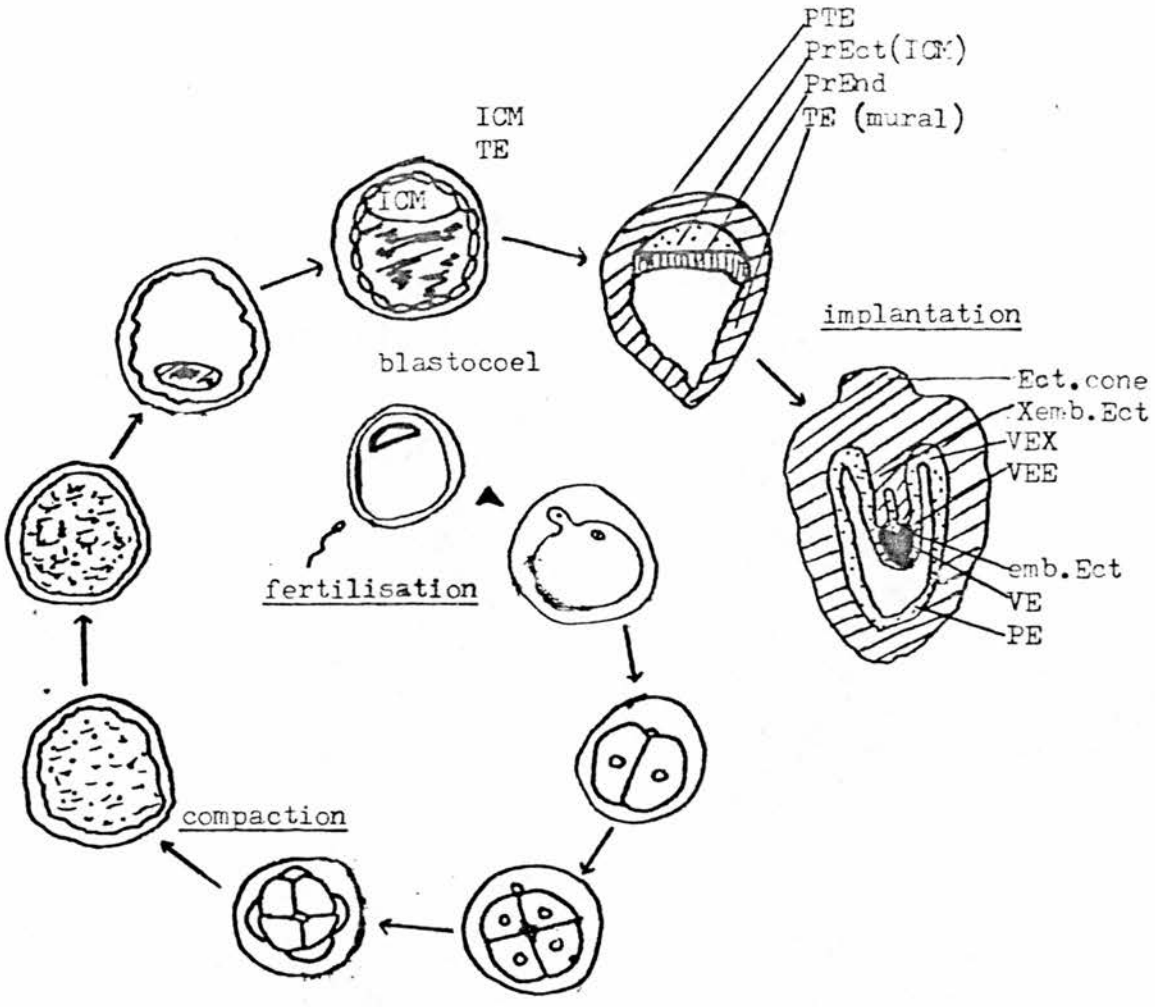
The maturation period of the oocyte lasts 14 days which time is probably a period of activity and preparation within the egg.Johnson [72] has shown that RNA synthesis is continuous up to the onset of Germinal Vesicle breakdown (GVBD) when this synthesis is terminated,although the majority of RNAs synthesised during this pre-ovulatory synthesis period appear to be retained over the next 7-9 hours before the start of ovulation.Environmental effects may include the interaction of the cumulus cells with the maturing oocyte and a hormone activity of cumuli between ovulation and fertilisation.Sulphated

Figure 1:1

FERTILISATION AND EARLY DEVELOPMENT OF THE MOUSE EMBRYO

Shows the development of the mouse embryo from fertilisation through the 2-cell and morula stages to compaction,formation of the blastocyst and development of the early egg cylinder after implantation.

ICM - inner cell mass
TE - trophectoderm
VE - visceral endoderm
PE - parietal endoderm
Ect.cone - ectoplacental cone
Xemb.Ect - extra-embryonic ectoderm
emb.Ect - embryonic ectoderm
Pr.Ect - primitive ectoderm
Pr.End - primitive endoderm
PTE - polar trophectoderm
VEE - embryonic visceral endoderm
VEX - extraembryonic visceral endoderm



Adapted from:

1) Johnson,M.H. Biol.Rev. 56 463-498 (1981)
2) Adamson,E.D. and Gardner,R.L. British Medical Bulletin 3 113-119 (1979).

Glycosaminoglycans,which are found in follicular fluid and as a component of extracellular matrix,can be shown to inhibit enzyme activity and therefore affect the synthesis of hyaluronic acid and other cellular processes in the maturing oocyte [34].This observation may reflect the operation of some form of developmental control.

Mouse embryogenesis begins,therefore,with the maturation of the female germ cell line,the"oocyte"and by its subsequent ovulation from the ovary,where the female mammal stores her entire stock of germ cells,and into the oviduct,which is the coiled connecting tube between the ovary and the uterus.Here the oocyte awaits fertilisation,which normally occurs in the ampulla tubae,the dilated uppermost loop of the oviduct [176].

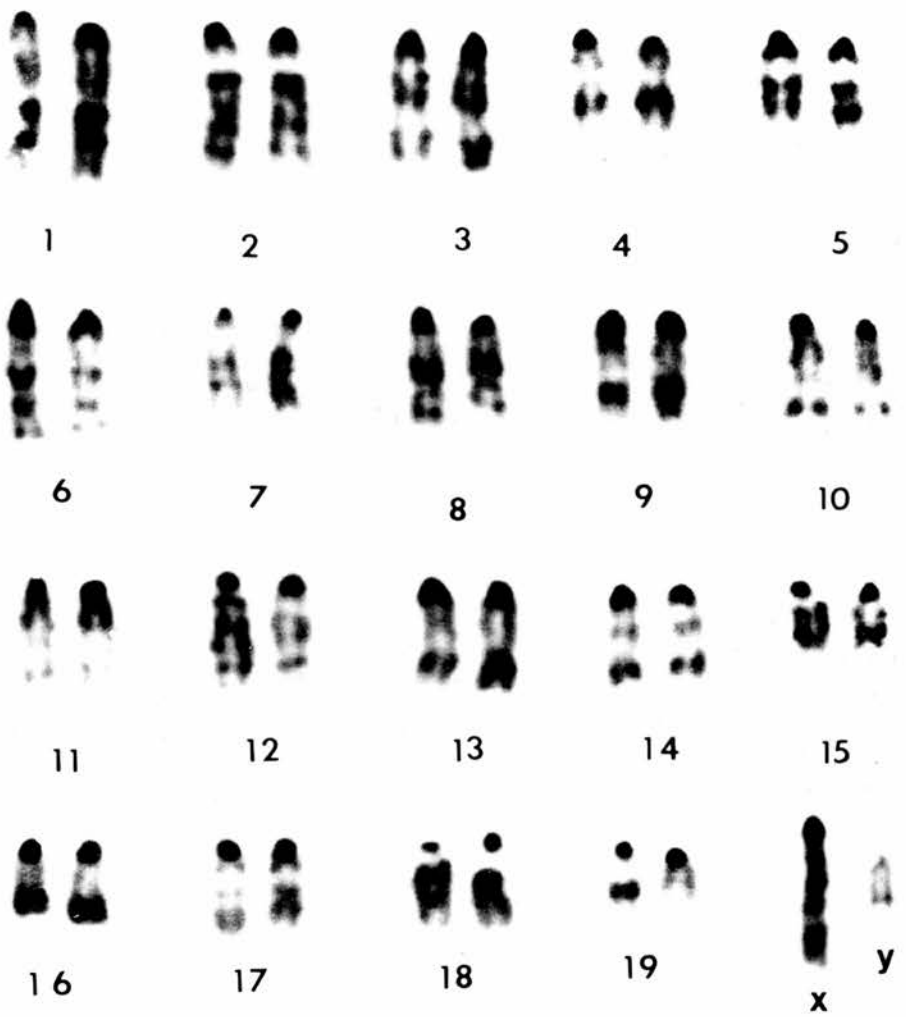
At this stage the chromosomes are arrested on the metaphase spindle [72] in meiosis of the second maturation division,a process which is only [34] normally completed after fertilisation.The nuclei of these cells therefore have a haploid (1n) complement of chromosomes,which in the mouse is 19 + X (the sex chromosome).Figure 1:2 illustrates the karyotype of a normal mouse somatic cell,which has been trypsin banded and stained with Giemsa.The 19 paired autosomes are all acrocentric,with deeply staining centromeric regions.The X and Y sex chromosomes of the mouse are very different from each other,the X being 2-3 times the length of the Y.

Parthenogenesis,which involves the development of the ovum without the stimulus of fertilisation has been achieved either spontaneously or by the use of an artificial inducer such as ethanol [I.5.3].The need for fertilisation in order to complete the 2nd meiosis and continue development has also been obviated using the experimental technique of oocyte fusion,using polyethylene glycol (PEG) [52],indicating a role in the mixing of either or both of the oocyte cytoplasm and genome.

The polar body from the first meiotic division may or may not be present but is usually cytolysed by the time that the oocyte has reached the ampulla.After ovulation the oocytes are surrounded by a clump of follicle cells,the cumulus,and there are normally 3-5 ova plus the cumuli in each ampulla.Although this number may vary from strain to strain.The number of ova ovulating at one time can be artificially increased by hormone injection in the experimental procedure known as superovulation [47,130] which exploits the

Figure 1:2 The Karyotype of Mus Musculus

The karyotype of the (male) mouse somatic cell has 19 paired autosomes,all of which are acrocentric and have darkly stained centromeric regions.The X and Y are clearly different with the Y being approximately 1 third of the length of the X. The female karyotype is the same except that two X chromosomes are present and the Y is absent



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hormonal regulation of ovulation by overstimulation of the follicles.

Superovulation is performed in mice by injecting the female with two hormones spaced 48 hours apart.House mice normally mate at around midnight and so in order to make ovulation time coincide with normal mating habits mice are usually injected at a time between 12 noon and 4 pm.The hormones involved are Gonadotrophin from pregnant mares serum (PMSG) which is injected first and human chorionic Gonadotrophin (HCG) which is injected 48 hours later and usually taken as the ovulation point.Mating,and therefore fertilisation,is usually taken to be 12 hours "post-HCG".

1.2.2. Fertilisation and Very Early Development

Before fertilisation can take place the sperm must penetrate the glycoprotein envelope,called the Zona Pellucida,which surrounds the egg.This is probably mediated by the use of trypsin-like proteases which are secreted by the sperm [143] and whose role has been indicated by experiments showing that the use of protease inhibitors can block fertilisation.The sperm also secretes other enzymes,for example Acrosin,but it has not yet been shown whether or not these enzymes also play a role in the attachment,penetration,or fertilisation of the egg.

Within 24 hours of fertilisation,the first cleavage division of the embryo takes place.The embryos are still in the oviduct,at this stage having just left the ampulla region.They then are moved down the second coil of the oviduct by means of a peristaltic contraction which appears to be exhibited by the lower portion of the oviduct [176].At the 2-cell stage the embryos are approximately 80-100 microns in diameter,consisting of the two cells of the first cleavage,which are of approximately the same size with large spherical nuclei containing 4-5 nucleoli and a finely granulated cytoplasm.Surrounding these two cells is the translucent Zona Pellucida.

The cytoplasm granulation has been used as a means of distinguishing between embryo cells of different genetic origin in lineage experiments [77] as visually distinct mutations of this phenotype are available.

Once fertilisation is effected,the male pronucleus approaches the female pronucleus and mitosis is begun to produce the diploid single cell embryo

[72].The expression of maternal mRNAs,such as that for the X-linked enzyme HPRT is initiated about 6 hours after fertilisation.These mRNAs continue to be translated up to the compaction stage [72].Proteins,rRNAs,mRNAs and probably also tRNAs are all inherited from the oocyte,therefore the newly fertilised egg is fully equipped to synthesise proteins without the need to transcribe its own genome.Therefore from fertilisation up to the mid 2-cell stage the embryo appears to rely entirely on the post-transcriptional modification of maternally derived gene products [72].Similarly,up until the implantation stage no division of mitochondria takes place,so that all the mitochondria in the cells of the early stage embryo were originally in the ovum.

1.2.3. Early Development of the Preimplantation Embryo - the Morula Stage Embryo

After the first cleavage division,the embryos continue to move down the oviducts towards the uterus,and over the next 24 hours undergo the 2nd,3rd and 4th,cleavage divisions giving morula embryos which are composed of approximately 8 to 16 cells.The cells during these stages are usually referred to as blastomeres.There is considerable interembryo variation and some morula embryos can have up to 25 cells after 2 days [176],this variation is probably due to differences in fertilisation times and appears to be at least partially genetically determined as some strains have a much wider range of stages after 2 days than do others [131].This stage has been called the "cleavage period"[113].

The changeover from the translation of maternally derived mRNAs,such as those coding for the enzymes HPRT and G6PD,to embryo derived mRNAs is begun at the 4-8 cell stages and has reached completion by the time that compaction of the morula has begun.This process coincides with a reduction in the requirement for many of these proteins [72].

At the late 8-cell stage the morula undergoes a process known as compaction,where the blastomeres maximise contact with their adjacent cells and it becomes more difficult to distinguish the individual cells from each other [176].This process is preceded by the formation of small gap and macula occludens junctions and complexes of these junctions which then develop with the formation of focal-tight junctions and gap junctions in conjunction with

compaction [95].This stage also marks the onset of the synthesis of cell surface Galacto transferase,which then continues through to the late blastocyst [146].

By the third day after fertilisation the embryos have mostly reached the mouth of the uterus and are at the post-compaction stage with approximately 16- 25 cells.Once in the uterus the embryo continues to divide,the outer layer of cells being now slightly larger than the inner layer of cells [73].

These cells have been shown to have different cleavage times from the division of the 8- cell stage onwards depending on position,the smaller cells on the inside of the morula tending to divide after about 14 hours while those on the outside which are larger have on average a division time of 12 hours [93].This result conflicts with earlier studies using radioactive thymidine which indicated that the inner cells divided more rapidly,but it is consistent with the development of trophoblast and ICM as the former contains many more cells at the blastocyst stage than does the ICM.

The 16-cell morula possesses two distinct populations of cells,the inside cells having a sparse,even microvillus distribution with uniform labelling of fluorescent ligand,while the outside cells were polarised with their exposed surfaces showing both heavy ligand labelling and a dense population of short microvilli [131].

The polarisation of morula embryo cells coincides with the onset of compaction at the late 8-cell morula stage.Cells of 4- and early 8-cell stage embryos show an even distribution of both fluorescent ligand and of microvilli.During the 8-cell stage the blastomeres exhibit a progressive restriction of microvilli to the outside,exposed surface of the embryo so that by the late 8-cell stage the outside cells were polarised with a dense population of short microvilli on their exposed surfaces and a sparser distribution of longer microvilli on their contacting surfaces,as shown by the scanning EM [133].This process is echoed by a similar polarisation of fluorescent ligand to those areas with a dense population of microvilli.

1.2.4. Blastocyst Development and Implantation

The embryo cells are not clearly differentiated and cannot be identified with certainty as trophoblast and inner cell mass cells until after the formation of

the segmentation cavity which occurs very rapidly.In strain C57Bl cavitation occurs at about 78 hours post fertilisation [176].However,recently [107] it has been shown that cavitation time is dependent on strain and may occur up to 20 hours later than this (MF1 derived embryos) with CFLP derived embryos cavitating between 83 and 93 hours post HCG [1.2.1].

The formation of the blastocoel marks the beginning of the blastocyst stage and is created by the formation of vacuoles in some of the morula cells which then discharge their contents to create the fluid filled cavity [113].This is first observed as a small,oval,translucent region located at the abembryonic pole and bounded by the attenuated processes of the mural TE.Occasionally this is split into two cavities within the single blastocyst. The blastocoel then continues to enlarge in a process known as expansion which takes several hours to complete,after this the blastocyst is in a fully expanded state and hatches out of the ZP.

By the 4^{th} day post fertilisation all of the blastocysts have reached the uterus and have cavitated,become fully expanded and have hatched out of the zona pellucida.The blastocysts then space themselves out along the length of the uterine horn,in preparation for implantation,and in most cases are already in close contact with the uterine epithelium.

The blastocyst embryo is made up of two distinct cell types these are the trophectoderm (TE),which at this stage comprises a single layer of flattened epithelial cells around the outside of the egg,and the inner cell mass which is a group of cuboidal cells within the TE,clustered at one end of the blastocoel [Figure 1:1] at the embryonic pole.These two cell types are the foundation for the divergence into extraembryonic and embryonic cells,which is the next stage of development [14].Both ICM and trophoblast cells contain nuclei with an elongated nucleolus and peripheral chromatin,additional nucleoli are also sometimes present [176].The nuclei of ICM cells are larger than the small more prominent nuclei of the TE.The number of cells varies but trophectoderm is in the region of 100 cells and the ICM 25-30 cells.

Implantation is estimated to occur at about day 4.5 after fertilisation, although there are notable differences in degree of development within the same litter [176],so that some embryos are just beginning implantation and are in close contact with the as yet undamaged uterine epithelium,while in other cases

there is already extensive erosion of the uterine lining.

The implantation procedure involves the erosion of the uterine epithelium to allow the integration of the blastocyst and usually starts at the lower half of the embryo with the trophoblast cells in this area becoming transformed into "trophoblast giant cells" as their nuclei enlarge and their cytoplasm puts out long slender processes.These cells facilitate uterine lining erosion by dissolving the membranes and they secrete large lipid droplets which may be involved in this process.The trophoblastic giant cells appear to be incapable of undergoing cell division and therefore do not generate any further cells.

The Primitive endoderm is by now distinguishable as a distinct layer of cells around the inside lining of the blastocoel adjacent to the ICM.These cells can be identified by their very dark eosinophilic cytoplasm.As the egg cylinder develops,the PrE further differentiates so that covering the ICM region is a lining of cuboidal cells which are the visceral endoderm,their free ends being densely populated with microvilli.At the opposite end of the blastocoel cavity,adjacent to the trophoblast giant cells,is a layer of parietal endoderm cells which form from cells migrating from the ICM around the inner wall of the blastocoel.This layer of cells secretes "patches" of extracellular matrix material which eventually forms a membrane between the trophoblast and endoderm cell layers and which is called "Reicherts membrane".

At the opposite end of the embryo the trophoblast cells multiply and become cuboidal forming a cap around the embryo which eventually becomes the ectoplacental cone [Figure 1:1].Ectoplacental cone cells stain extensively with H & E and are easily distinguished from the ICM cells.At the same time the ICM enlarges considerably and bulges into the blastocoel cavity to form the egg cylinder.At this stage these cells,derived from the ICM,are usually called the Primitive Ectoderm,Embryonic Ectoderm or more accurately the Epiblast.These cells are the precursors of the foetus itself.

Ultrastructural and monoclonal antibody analysis of the component cells of the Blastocyst to Egg Cylinder stage embryo has revealed several distinguishing features between the various cell types.ICM cells show no evidence of differentiation on EM pictures.They have large regular nuclei with more darkly shaded nucleoli,2-3 in number and very few cytoplasmic features apart from a small number of elongated mitochondria with lamellar cristae.They express

SSEA1 [Table 1:1] in their cytoplasm and like Primitive Ectoderm and embryonic ectoderm they also express the Forssman (M1/22.25) antigen [169].Unlike the rest of the implantation stage embryo cells,ICM cells do not have intermediate filaments [41],nor do they synthesise transferrin [2],although this latter is made by Pr Ect cells from day 7 onwards.

EM of endoderm however has revealed several differentiated features which enable the identification of individual endoderm cell types.PrE cells have an extensively developed branched RER with intracisternal accumulation of matrix [33],a feature which they share with the PrE of all other mammalian embryos investigated.Their mitochondria become more rounded with tubular,rather than lamellar cristae.These morphological changes are accompanied by the appearance of intercellular junctional complexes at the apical sites of membrane apposition,so that the PrE is arranged in a simple membrane epithelium.

PE however,is made up of individual stellate cells which,although they continue to show the dilated RER characteristic of PrE cells,have lost all junctional association with other cells.Their outer margin characteristically has only a few microvilli.PE cells are associated with large amounts of basement membrane,which like the PrE cells,they synthesise.Because of the large amounts of matrix which they secrete,the cells of the parietal endoderm tend to have a comparatively slow growth rate and divide much less frequently than the cells of the PrE or VE.

In contrast VE cells are cuboidal,closely associated with one another,joined by apical junctional complexes and forming a continuous epithelium with numerous short microvilli on the apical surface.The cytoplasm of these cells develops both large and small vesicles,most of which are electron-lucent or whose contents were washed out during processing so that they appear as holes under EM.VE cells,like TE,express the antigen SSEA1 in their cytoplasm,but not on their cell surface.Endogenous peroxidase [9], Transferrin [2] and AFP have all been shown to be synthesised exclusively by VE cells, from the 6^{th} day post fertilisation and therefore could be regarded as markers for VE differentiation,although transferrin is also synthesised by Pr Ect from day 7.Endogenous peroxidase activity has been associated with hormone synthesis,although it could also have a bactericidal or viricidal effect.In the VE peroxidase activity is always localised inside or close to the many apical

Table 1:1 Cell Surface Markers Found on Early Mouse Embryo Cell Types and on EC cells

Summarises the results of Monoclonal and poly- clonal antibody experiments with various cells of the preimplantation and early post-implantation mouse embryo and of similar experiments using mouse teratocarcinoma derived cells (EC cells)

NT - not tested

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Cell Type	Histocompatability markers	Other Markers				
		5D4	M1/22.25	SSEA1	SSEA111	2C5
Trophoblast	-	-	-	-	-	-
Parietal endoderm	-	-	+	-	-	-
Visceral endoderm	-	NT	+	+	+	NT
EC cells	-	+(-)'	+(-)'	+(-)'	-	+(-)'
Primitive endoderm	-	NT	+	-	-	NT

notes:

' - EC cells have been generally shown to have these markers but in all the cases indicated variants have been isolated which do not have these markers (1,2,3).

vacuoles which are also a feature of these cells.

Mural TE,syncytiotrophoblast,consists of large cells 40-50 μm in diameter with abundant cytoplasm and a deeply indented or segmented nucleus.TE of the early blastocyst expresses SSEA1 antigen,low level H-2 and B-2 microglobulin [175],alkaline phosphatase,Forssman (M1/22.25) antigen and has visible cytokeratin filaments [85] which react with the cytoskeletal protein specific antibodies Endo A and Endo B [175].They all contain a few endocytic vesicles (lysosomes) and also characteristically contain glycogen granules,especially under the influence of oestrogen,and have the potential for steroid synthesis and produce the enzyme Beta Hydroxy Steroid Dehydrogenase (BHSD).Both ectoplacental and mural TE show the transient presence of H-2 antigen [1] which then disappears,together with SSEA1,as the embryo develops.

1.2.5. Post- Implantation Development and Formation of the Germ Line

By 6 days the egg cylinder has fully developed and is divided into embryonic and extra-embryonic regions,later to form the foetal tissues and foetal membranes respectively and the differentiation of endoderm is complete.The proamniotic cavity first develops at this stage first appearing in the embryonic region and later extending to extra-embryonic tissues. Coinciding with the expansion of the proamniotic cavity at day 7 is the first appearance of mesoderm,located in the primitive streak region.

The differentiation of endoderm,begun during the early egg cylinder stage,continues with the division of VE into embryonic (VEE) and extraembryonic (VEX) endoderm at day 7-8.VEX is found underlying the extraembryonic ectoderm and does not synthesise AFP,whereas the VEE does synthesis AFP and is found in association with the embryonic ectoderm [61]. Endogenous peroxidase [9] continues to be present in VE cells up to the 9^{th} day and is not found in either mesoderm,ectoderm,ectoplacental cone or TE tissues.

Extraembryonic yolk sac endoderm cells (VEX),in common with the cells of other extra-embryonic tissues, normally undergo a non- random inactivation of the paternal X-chromosome and display an abnormal ability to facilitate gene transfer which probably indicates that X-chromosome inactivation in these cells differs from that in normal adult mouse tissues and from embryonic cells at

the DNA level [82].

Intermediate filament expression studies have found that cytokeratin is expressed in all cell types by this stage and in PE cells between 8.5 and 13.5 days vimentin is also found [85].

Germ cells first appear in the genital ridge of the 12.5-13 day old embryo as primordial germ cells,they show a positive reaction with M1/22.25 (Forssman antigen) from the time of their appearance until they are fully formed [169].Soon after entering the genital ridge mouse primordial germ cells take either a male or a female pathway of development,depending on the sex of the embryo, and by day 14-15 they are identifiable as oogonia or spermatogonia [107] and have lost the cell surface reaction with the Forssman antibody,although other cells in the genital ridge, not related directly in lineage,do become positive to M1/22.25.These positive cells orssman antigen positive in the adult mouse testis.

At the 14 day stage the cells of the foetus behave like those of the adult mouse in that the inactive X-chromosome is no longer efficient in gene transfer,in contrast to the behaviour of the non-randomly inactivated X-chromosomes in the cells of the early embryo,which are capable of gene transfer [82].

1.3. Control,Determination and Cellular Differentiation

Research on the various biochemical and genetical changes taking place over the early preimplantation stages of mouse development were reviewed in 1979 by Adamson and Gardner [1] and then by Johnson [72] and Magnusson & Epstein [96] both in 1981.

Experiments in which male or female nuclei were implanted into haploid,parthenogentic eggs have provided evidence for the genetic imprinting of the genome during gametogenesis [170].Results showed that the egg cytoplasm was capable of supporting development to term but that this would not occur without the presence of both male and female genomes.The presence of the male genome seems to be essential for the normal development of TE and extraembryonic membranes at the late blastocyst and egg cylinder stages.This may in part be explained by the preferential

inactivation of the paternal X- chromosome in these tissues [171] which may indicate an as yet poorly understood mechanism of embryonic/extraembryonic tissue differentiation.

The importance of translational control in early development is not fully understood but it seems likely that mammalian organisms,being smaller and more able to interact with outside sources of nutrition, would rely less on the pre- existence of a large stock of ready-transcribed gene products than do amphibian embryos such as Xenopus,which are externally fertilised and therefore have to be autonomous throughout their life cycle [182].Xenopus embryos therefore contain materials at the 30,000 cell blastula stage (10 hours,21°C) which would take the diploid zygote approximately 16 years to make.

Mammalian embryos can therefore allow more flexibility in their development and are probably only dependent upon translation as a major form of their developmental control for a very short period of their existence,up to the mid 2-cell stage.One thing however,which does seem clear,is that no one form of control is in operation at one time and although at most stages there are control mechanisms which seem of overiding importance in determining future events,it is perhaps in the changeover and interaction of these various control mechanisms that lies the greatest potential for an experimental approach to development.

1.3.1. Biochemical and Genetic Events in the Oocyte and Very Early Embryo

The building up of food resources is well known in animals which undergo fertilisation and/or development outside the mother,in which the egg is required to be completely autonomous throughout its development,but has a more obscure role in mammalian development in which the embryo is nurtured inside the mother's uterus until it is fully formed.It seems entirely reasonable however,that the mouse egg,like that of other species such as Xenopus,should inherit both a set of proteins critical to early development and a capacity to synthesise new proteins [72].

It seems clear that the preovulation phase of oocyte maturation is a period of preparation and synthesis,during which time the egg is equipped with a supply of maternally derived proteins,RNAs and possibly other gene products in

preparation for fertilisation and early development of the embryo.Some 7-9 hours prior to ovulation this synthesis apparently ceases,coinciding with the onset of germinal vesicle break-down (GVBD),and the products of this period of activity appear to remain inactivated and stable throughout ovulation and until after fertilisation. It would seem that some control mechanism is set in operation by the onset of GVBD [84] which causes the inactivation of maternally derived gene products,possibly by some form of physical packaging [72].

The process of fertilisation causes the release of these gene products.The mechanism involved is not known but it seems more likely to be one of derepression,possibly by the removal or alteration of a suppressor which is preventing translation (like the histone related translation supressing proteins in the Sea Urchin) post-translational modification, rather than oocyte activation [184].The existence of parthenogenesis which occurs in most mammals,including the mouse,indicates that fertilisation is probably most important for the continuation,rather than the initiation of development.

At the early preimplantation stages the mouse embryo is only 70-100μm in diameter and contains approximately 0.5ng RNA,23ng protein and 20-1000 fmols of free individual amino acids [72].Analysis of transcriptional activity in the cells of the 1-2 cell embryo is largely involved with the extent to which inherited maternal information is acting and to what extent the embryonal genome itself is being transcribed.

From fertilisation to the mid 2-cell period the embryos appear to depend largely on the interaction with their environment and pre-existing maternal enzymes and RNAs for the determination of events.Evidence for this has been furnished by studies on transcription levels [72] and studies measuring the generation of active RNAs and de novo synthesised proteins.The mouse embryo therefore appears to exist entirely on maternally derived gene products for the first 27 hours (up to the mid 2-cell stage) of its life,after which there is a gradual introduction of embryo transcribed material,HnRNA and mRNA,as large numbers of embryo genes switch on during the mid 2-cell stage [72]. The embryo genome is shown to have some effect from the late 2-cell stage onwards when homozygous lethal mutations of the development-related t-locus,first begin to have effect.The first defects expected to reveal themselves at this stage are therefore inherited mutations in genes which

affect transcription and translation.

Prolongation of the maternal effect after this stage must,therefore rely on the survival of proteins previously synthesised on maternal templates,or on the survival of small populations of maternal mRNAs.In fact it has been shown that there is very little incorporation of new uridine triphosphates (UTP) before the 8-cell stage,thus indicating that there is little or no *de novo* rRNA synthesis before this stage. The relatively simple medium in which preimplantation embryos are able to grow indicates that few metabolic precursors from outside of the cell are required for the continuation of growth of the early preimplantation stages [96].

The provision of new ,or destruction of old subsets of mRNAs may mark critical points in the differentiation and commitment of embryonic cells.Most maternal mRNA ceases to be active after the 2-cell stage.The largest qualitative change in protein synthesis occurs between the 2- and 8- cell stages of the morula and not at the morula to blastocyst transition [96].

Most embryos exhibit a 2-cell block to in vitro growth and will not grow *in vitro* if removed from the *in vivo* state before this stage.The 1 and 2 cell embryos of certain inbred strains and F1 hybrids (C57Bl/CBA) are not restricted in this way and will grow entirely *in vitro* to at least the egg cylinder stage. Recently [117] it has been shown that this *in vitro* block can be overcome ,by taking small amounts of cytoplasm from the embryos of mice which do not exhibit the 2-cell block and injecting this into the 1- or 2- cell embryos of randomly bred (MF1) mice.These embryos will then develop in vitro up to the 4- or 8- cell stages at least.Injecting cytoplasm from 2-cell embryos has a greater effect than that obtained by the injection of cytoplasm from 1-cell embryos.

This *in vitro* 2-cell block can also be overcome by the fusion of an MF1 (2-cell blocked) oocyte with an oocyte from a F1 hybrid not displaying the block [52].This technique allows the development of the oocyte to blastocyst stage and even beyond in some cases. Therefore the independence of the 2-cell block from the fertilisation process appears to indicate the involvement of some aspect of the maternal provision of gene products rather than any interaction with the sperm.

This phenomenon is an indication of epigenetic interaction of mouse gene(s)

with the oviductal environment which appears to have been overcome by a mutation in the inbred strains which possibly allow the provision of a metabolite,RNAs,or other gene product by the embryo itself instead of requiring some interaction with the maternal environment and/or genome.Alternatively a mutation in an inhibitory gene could be in operation.

Evidence for the early involvement of at least some paternally derived genes is demonstrated by the detection of paternal variants of Gpi-1 [23] in early mouse preimplantation embryos.Although some enzymes (e.g.HPRT and G6PD) appear to be expressed in the maternal form from 6 hours post-fertilisation up until the compaction stage of the embryos development when the synthesis of embryo forms of the enzymes starts to take over.

1.3.2. Early Determination Events in the Morula Embryo

The development of the mouse embryo between the 2 cell and blastocyst stages has been extensively studied,both because it is the most accessible stage to *in vitro* culture and observation and is a period during which the cells of the embryo are undergoing rapid proliferation and many changes.It is during this preimplantation period that the first differentiations occur,producing cells which are "determined" and therefore apparently restricted to one or a few pathways unlike the totipotency of the cells from the first few cleavages.

Research over this period has mainly concentrated on the attempt first to discover the extent of determination of the individual cells of the morula embryo and second,to show what mechanism or mechanisms are influential at this stage.To some extent the two questions are inextricably linked and the elucidation of the first can lead to the ruling out of one theory and the favoring of another.

Early workers like Dalcq [28] proposed that cell differentiation was due to a preformed asymetry,present in the cytoplasm of the oocyte,such that differences (and therefore determination) arose automatically with each cleavage of the embryo.This would necessarily mean that the cells of the embryo are determined from the oocyte stage,unless some system involving the asymetry caused by sperm entry were invoked.

Recent research work,however,seems to point towards the cells of the cleavage

stage embryo remaining highly labile as to their fate,until at least the late morula stage.Much of the early evidence for this conclusion arose from experiments involving the isolation of single blastomeres,which where then cultured *in vitro* and shown to be capable of producing complete blastocyst embryos [174].This method was however limited by what has been described as a "Biological Clock" [113] within each blastomere cell which causes the daughter cells of isolated 8-cell stage blastomeres to attempt to cavitate and form a blastocyst,even with an insufficient number of cells to allow formation of a normal blastocyst.

Therefore,more recently,more sophisticated experiments involving embryo reconstruction have been devised to investigate the potency of late morula and early blastocyst cells.In order to study the potency of 4- and 8- cell stage blastomeres into the post-implantation stage,Kelly [77] utilised the genetically dependent variation between cytoplasmic granule content of blastomeres,by making up chimaeric composites containing "same stage" blastomeres from two different genetic origins,arranged in 1+3 "quartets".Examination of these composite embryos on recovery on the 10th day of pregnancy or at term revealed extensive donor cell contribution in all areas of the mouse,yolk sac and trophectoderm,both from 4- and 8- cell blastomeres,indicating that the cells of the mouse embryo are totipotent at least up to the morula stage.

The demonstration that the cells of the early mouse embryo were not determined at least up to the pre-compaction stage is contradictory to the idea of preformed differences within the egg itself and has led to the development of alternative theories which seek to explain determination as a result of biochemical or genetic influences.Therefore the embryo would seem to be "programmed" and rather than being actually physically preformed from the ovum stage,the early stages are therefore a means of creating these differences,possibly by the use of pre-existing RNAs or biochemicals [see 1.3.1], by a genetically determined programme of events or by some epigenetic interaction of these forces which might operate either within or between the cells and also with the maternal environment.

The relative ease with which these early stage embryos can be cultured *in vitro*,and the fact that the cleavage stage embryos are more easily cultured outside the maternal environment than any other stage,would certainly indicate a large degree of autonomy and seems to belie the importance of the maternal

environment at least at this stage.However,the embryos do grow more slowly *in vitro*,although the demonstration that *in vitro* cultured preimplantation embryos can be replaced into the uterus and then develop normally to term [174],does indicate that nothing of major developmental significance is lacking as a result of the absence of the maternal environment at this stage.However the very need for,as yet undefined,culture medium shows that external nutrients and probably also growth factors and other chemicals,adequately supplied by animal serum and normally produced by the mother are also required by the developing embryo.

Much evidence has been recently accumulated which suggests that positional information may be an important process in the early stages of determination.Experiments on the polarity of cells,with respect to position of microvilli and fluorescent ligand binding,situated on the outside and inside of morula stage embryos [133,73,74,192] and distribution of cortical myosin [156] have shown that it is possible to alter and determine various features of these cells simply by isolating them or otherwise changing their position in relation to the other blastomeres.

Johnson and Ziomek [73] have shown that polarised morula embryos [section 1.2.3.] can be reconstructed experimentally from isolated 8-cell blastomeres in which the polarity can be induced by controlling the cell-cell contacts of each blastomere *in vitro*.The ability to induce polarity in these blastomeres appears to develop during the 2-cell stage and contact with the membranes of cells from late 2-,4-,8- and 16-cell stages can all induce polarity in 8-cell stage blastomeres within 3-5 hours.Once formed this polarity appears to be stable.Unfertilised and newly fertilised eggs were found to be incapable of polarity induction,as also were early 2-cell eggs.Multiple contacts affected the orientation of the axis of polarity and cells which were completely surrounded failed to polarise,therefore mirroring the situation in the normal late 8-cell morula.

Sobel [156] gives evidence of polarisation of cortical myosin in contacting cells from as early as the 2-cell stage,which correlates with the onset of the ability to induce polarisation of microvillus distribution in 8- cell blastomeres.The loss of myosin from regions of cell contact correlated with the development of cell contacts which were stable to Triton X-100 extraction of lipids and protein from the embryos,although pronase and acid salt solutions were shown to

interfere with the stability of these contacts.

However,there is no evidence that the polarisation of myosin in 2-cell embryo cells is permanent,and evidence on cell determination of these stages would tend to suggest that they were developmentally labile,therefore it seems likely that the first overt differentiation of the mouse embryo is at the 8- cell stage when the individual blastomeres become morphologically polarised along a radial axis [73].

However,although it has been shown that regions within an individual cell are able to differentiate from each other,Kelly [77] has shown that isolated blastomeres are totipotent up to the 8-cell stage,it would seem therefore that these cells are not totally committed in one direction and are able to resurrect total potency if they are experimentally forced to recombine with other blastomeres in alternative positions.This is possibly an indication that cell communication is an important developmental factor at this stage and it is at this time that both tight and gap junctions are formed between different blastomeres and that some forms of communication at least can be demonstrated to be compartmentalised,with the use of fluorescent dye injection.[89] and ionic coupling experiments.It has also been shown that if compaction is blocked by the use of antibodies then the morula is not capable of proper formation of the blastocyst and therefore of further development.Therefore compaction is apparently a crucial step towards determination and development of the mouse embryo.Decompaction is also occasioned by the exposure of morulas to Ca^{2+} free M16 medium containing BSA for about 20 minutes and also by the addition of Cytochalasin D to this medium [131].

Reaggregation experiments to investigate the potency of 16-cell morula blastomeres [192], however indicate that determination at this stage is more developed,although there still appears to be some lability.16-cell morula blastomeres were isolated *in vitro* and typed as either smaller or larger cells,to indicate either an inside or outside origin respectively.Labelled large cells contributed mainly trophectoderm if placed on the outside of aggregates and when placed on the inside or randomly they always produced at least one trophectodermal offspring,but in some cases also contributed to ICM.Smaller cells,on the other hand would generate mainly ICM if placed on the inside and if placed on the outside of aggregates or randomly would generate,ICM

alone,trophectoderm alone,or both tissues.It would therefore seem that the outside cells are more determined than the inside cells so that phenotype is of major importance in deciding the fate of the larger cells,but positional information is the main deciding factor for the smaller cells which were derived from the inner part of the 16-cell morula.

The timing of the changeover from maternally derived to embryo derived gene products and the reduction in quantity of enzymes such as HPRT and G6PD [1.2.3] with the onset of compaction,polarisation and gap-junction formation may reflect the existence of some common mechanism directing this changeover and perhaps marking the end of one type of developmental control and the start of another,this latter possibly depending more on cell-cell interaction and communication than translation of pre-existing maternal gene products.

Prior to ovulation oocytes communicate with interacting follicle cells (cumulus oophorus) via gap junctions.With the onset of ovulation they become progressively uncoupled so that the mature oocyte and early developing embryo do not communicate via gap junctions at all.This was shown by Lo and Gilula [89] who measured the passage of Fluorescein (MW 330) and Horse Radish Peroxidase (HRP) (MW 40 000) and also the ionic coupling between cells of embryos at different stages.The HRP molecule is used as a control as it is too large to pass through a gap junction but is able to be transferred via cytoplasmic bridging.Transfer was shown to be only possible between adjacent sister cells in 2- 4- and early 8- cell embryos indicating that the only mode of transfer at this stage were cytoplasmic bridges.Junction mediated communication was first detected at the late 8-cell stage both by ionic coupling and fluorescein,although HRP was not transferred except in a limited way via cytoplasmic bridging between sister blastomeres.

The asymetry of cell contacts in the 8-cell embryo,together with the stability of polarisation through cell divisions,creates a mosaic structure within the morula cells which is conserved and elaborated on during the 16- and 32-cell stages [72].These changes are therefore cumulative but reversible and represent a consolidation of determinative events,similar to that observed up to the mid 2-cell stage and that during the cleavage period from 2- to mid 8-cell stage.It has been suggested [72] that early embryo development is effected by a series of cycles with a periodicity of approximately 27 hours during which time

developmental changes are accumulated and that the boundary between one cycle and the next constitutes a period of commitment at which reversal of that stage of development becomes no longer possible.

During the transition from morula to blastocyst there is little inside/outside cell mixing,which could indicate the initiation of a new mechanism of physical restraint which enables the enhancement of already initiated differences between these cells.

1.3.3. Cell Determination in the Blastocyst Embryo

At the blastocyst stage there are two distinct cell types,the ICM and the Trophectoderm,which appear to be fully differentiated from each other.However the extent to which this process is irreversible is in some doubt and recent evidence has indicated that there may still be a considerable amount of lability in the fate of these cells ,particularly the ICM,which has been shown to be capable of contributing in small numbers to trophectoderm and placental tissues [137].Rossant and Croy have shown that,using isozymal and *in situ* genetic markers,about 70% of the 13-15 day placenta is TE derived,30% maternal (spongioblast) and 4% (the labyrinthine TE region,forming foetal blood capillaries and endodermal sinuses) from the ICM.Duval,however has already suggested that the endodermal sinuses are formed by the retraction of PE and VE into the placenta at the 8-9th day stage of development [61].

The finding of some unusually high ICM contribution to placenta in a few reconstituted blastocyst experiments using ICM's from early 3.5 day blastocysts [137] is less easily explained and suggests that these ICM cells had contributed to the trophectoderm layer of the blastocyst.

Evidence that the differentiation of TE is more advanced than that of ICM is indicated from several sources,Sherman et al [151,45] showed that approximately 1/5th of cells isolated from the 16-cell morula embryo could differentiate into trophoblast giant cells indicating that the fate of these cells was already determined along the TE pathway.The generation of these large polyploid cells expressing specialised enzyme activities such as BHSD and Alkaline phosphatase,and producing plasminogen activator in large amounts requires neither cell-cell contacts ,cell division, nor other developmental events such as hatching from the ZP,TE outgrowth and implantation,nor is their

differentiation dependent on maternal environment.Although Gardner and Johnson [44],using dissected blastocysts,found that the presence of the ICM was essential for the proliferation of TE both in vitro and In Vivo.As discussed in the previous section [1.3.2.] Ziomek and Johnson showed that the fate of outside cells of the 16- cell morula was more dependent on phenotype than on position within the embryo.

Therefore although contribution to the embryo by trophectoderm has not been comphrehensively ruled out,and may yet even prove to be possible,it would seem that the "differentiation" of the morula into TE and ICM cells once considered to be an irreversible divergence,may prove to be far more flexible,with the TE differentiating as a side shoot from the more labile ICM cells with considerable leeway for contribution of one cell type to the other lineage.

The first characteristic of the differentiation into TE is the acquisition of the capacity to envelope cells,and this develops very early in TE or in outside cells from isolated ICMs [25].At this stage gap junctional communication between TE cells is evident as is junctional communication amongst ICM cells and also between TE and ICM cells which are in contact [89].The differences between mural and polar TE could be explained by the action of physical mechanisms so that mural TE cells are stretched by the blastocoel and are not in contact with the ICM,so that their mitosis is reduced or prevented by lack of interaction with ICM cells but also by physical shaping so that the cells become unsuitable for cell division to occur very easily.Polar TE on the other hand proliferates,its cells becoming cuboidal and in a position to interact with the ICM,and eventually forming the ectoplacental cone.This is supported by both Gardner and Johnson [44] who showed that the presence of ICM was necessary for TE proliferation both *in vivo* and *in vitro*.In the absence of ICM cells the TE formed giant polyploid cells and did not divide,thus behaving in a way analogous to the abembryonic mural TE.

Experiments with dissected blastocysts [44] and isolated ICMs [160,44] with synchronised blastocysts [25] and reconstituted chimaeric blastocysts [137] have all tended to support the view that early embryo cells seem to drift towards determination rather than undergoing one irreversible event at a preset stage of their development.The considerable heterogeneity as to cell numbers both inside and outside the blastocyst,and in cell cycling and cavitation timing

found by Chisholm et al [25] may provide some clue to the confusion surrounding this problem.It was found that if embryos were synchronised according to their age post-cavitation rather than by hours after HCG injection,then with respect to cell numbers there was much less heterogeneity at this time with newly cavitated embryos having a cell number of between 28-35 cells and with the majority of outside cells in the 6^{th} developmental cycle,slightly ahead of the majority of inside cells.The latter have been shown to have a longer division time than the outside cells from the 5^{th} cycle stage [93].After cavitation however the evidence of intraembryonic variation of synchronised embryos was substantial and may indicate that the microenvironment of individual embryos may have a powerful influence at this stage of development.

Investigation of the developmental potential of post-cavitation synchronised embryos showed that the majority of isolated ICMs taken from blastocysts between 2 and 12 hours after cavitation showed the ability to produce trophectoderm,as measured by the generation of cells showing fluid accumulation and formation of blastocyst-like vesicles,within 24-48 hours of the start of the culture.This timing of vesicle formation allows the distinction between TE and endoderm formation as the latter would not be expected to form until 48-72 hours after the initiation of the culture.

The cells generating TE differentiation were those on the outside position of the ICM and the general trend was a reduction in the amount of TE generated with an increase in endoderm formation which correlated with an increase in age of the ICM.Although $2/3^{rds}$ of viable ICMs from 12 hour post-cavitation embryos still generated some TE cells and only $1/3^{rd}$ generated endoderm alone.A small proportion of ICMs generated a mixed population of TE and endoderm structures.These results were all confirmed by an analysis of their specific ultrastructural features,which indicated that the portion of TE cells present may be even higher and of endoderm cells slightly lower than suggested by LM analysis.

A series of experiments from Hogan and Tilly [60] using isolated ICMs separated from the blastocyst *in vitro* at different times of development,also support the gradual determination of ICM cells during the 3.5- 5.0 day period.So that the contribution to TE gradually diminishes with the increase of contribution to endoderm,this process correlating with the advancement of

developmental stage of the ICM when it was isolated.

Experiments which looked at the influence of orientation on the development of mouse ICMs *in vitro* [190] have shown that egg cylinders developing from mouse blastocysts placed in culture with the ICM in the lower position close to the plastic culture surface,will develope to more advanced stages (up to 8.5 day stage equivalent of (*in vivo* embryos) *in vitro* than will those placed in the upper lateral or upper position indicating that TE prevents growth of downward growing egg cylinders beyond a certain stage,whose development can however be prolonged by reorientation.This result indicates the importance of position in the development of early mouse embryos,although this may simply be an artifact caused by the physical restriction to growth imposed by the plastic culture surface.

The general conclusion of recent data on the potency and fate of mouse blastocyst embryo cells therefore seems to be that,although the fate of cells is normally predetermined for the majority of cells at this stage,the primary mechanism at this stage appears to be one of cell position and cell-cell interactions,rather than of altered phenotype,so that the cells of the blastocyst embryo,particularly the ICM,are still totipotent until well after cavitation.There also seems to be a gradual restriction of this potency as the ICM cells become older,so that the cells of 3.5 day stage ICMs will form TE much more readily than those of later stage ICMs.Therefore the ICM cells become "committed" at around the 6^{th}-7^{th} cell cycle,rather than around the 5^{th}.So that this event is after the blastocyst is fully expanded rather than occuring during expansion as suggested by Handyside and Barton [55].In concurrence with this,it appears that committed and uncommitted ICM cells can coexist in the same blastocyst so that in outgrowth experiments [131] the uncommited ICM cells can form TE but the commited (PrEctoderm) cells generate endoderm,which is compatible with the proposed relationship between cell cycle and commitment.

Under normal circumstances it would seem that the majority of blastocyst cells do not fulfil this potential and continue to develop along the pathway determined for them by their position,but even in the normal embryo it would seem that there is a certain amount of interchange between cell lineages as indicated by the finding of ICM derived cells making up a small portion of the placental tissue in chimaera experiments [137],although these are obviously not entirely normally developing embryos.

These findings indicate a possible mechanism for the initiation process of determination which would seem to be generated by a gradual physical restriction of totipotent cells so that their fate is restricted initially not by their genetic potential but rather by their access to specific microenvironments which then may interact with their genome to cause an actual phenotypic restriction of their potential.

Implantation and the restriction in potential of most ICM cells occurs during the same period,4-5 days post fertilisation,as the phenomenon of size regulation [113].This process was first observed in experiments in which giant -size composite blastocysts were placed *in utero* and observed during implantation.Double sized embryos were seen to adjust to normal size shortly after implantation at the early egg cylinder stage.

Although these experiments involved an abnormal situation it is thought,although not proven,that the same mechanism(s) is involved as that which regulates a species specific range of size normalcy for embryos in the natural situation.

Because of this coincidence of events,it has been suggested that embryo-determination and size regulation are interrelated.It has been proposed [113] therefore that the embryo may be derived from a very small constant number of primordial embryo cells in which,regardless of ICM size,becomes activated an"Embryo determining" loci.Mouse chimaera experiments suggest that at least 2,but possibly as few as 3 ICM cells may form the core of embryo primordial cells [113].Other physiological and extrinsic factors,such as uterine crypt size,could then regulate the proliferation of extraembryonic components.

In support of this hypothesis,Gardner and Johnson [44] have concluded that determination,defined as a progressive restriction in the potency of a cell,which is a general feature of embryogenesis,tends to occur in small populations of cells,is dependent on the position of cells relative to other cells in the embryo or tissue and involves a choice between two pathways only.This choice tends,in extraembryonic cell types to be closely followed by overt differentiation,whereas more flexibility is generally retained in the potency of those cell types destined to become the embryo.

There are many possible alternatives and combinations of this theme,including the possibility that the cells become self-limiting by some feedback

mechanism.Handyside [pers.comm.] has suggested that it is endoderm formation and anchorage dependence on the basement membrane which regulates size at this stage.

It seems likely,in any case,that the initiation of this process is random in that,until one cell undergoes this change and thereby introduces regulation of all other cells, one ICM cell is marked out to be the progenitor of the embryo.

1.3.4. Endoderm Differentiation

The generation of a layer of Primitive Endoderm cells at the blastocoel/ICM interface heralds the start of endoderm differentiation.After the ZP has disappeared,PAS staining reveals intense red granules within the trophoblast cells,while some ICM cells bordering the blastocoel also stain in this way,possibly indicating the first primitive endoderm cells [176].The two cell layers,PE and VE which are generated from these precursor cells do not actually contribute to the foetus itself,as they are discarded at birth [61] but their growth and differentiation represent a process which seems to be a recurrent theme in the process of embryogenesis itself.That of the generation from a common precursor cell,in this case PrE, of two cell types of restricted potential by some descision making process .Their differentiation is manifested both by heritable changes in gene expression,illustrated by differences in secreted proteins such as AFP which is secreted by the VE but not normally by the PE which unlike VE lays down large amounts of basement membrane,and by differences in cellular morphology,VE cells are organised into an absorptive and secretory epithelium,while PE cells are individual and migratory .

Cellular homologues of certain viral oncogenes [61] are differentially transcribed in extraembryonic and embryonic tissues,during this stage of development.This observation may reflect either the initiation of a further stage of developmental control or may be the result of such control.

PrE cells have been conclusively shown to contribute to both PE and to VE by injection of small numbers of genetically marked PrE cells into a host blastocyst [45].As the founding population of the PrE is very small,only around 20 cells,very little information other than this is available.It has been shown that PrE synthesises fibronectin and that during *in vitro* culture,once isolated ICM has developed a continuous endoderm layer the ICM cells no longer show

surface staining for fibronectin suggesting that fibronectin is probably secreted in a polarised manner between the two cell types,forming a precursor to the basement membrane.One possible mechanism for the generation of differentiation in endoderm is the gradual uncoupling from TE which has been shown by dye injection experiments.So that endoderm cells maintain gap junction communication with each other but do not communicate with TE [89].Although this process is certainly not completed at the PrE stage.

The cell division time of PE cells is very slow and possibly,during endoderm colonisation of the blastocoel,PE cells are recruited from the VE or residual PrE layer as well as being generated by PE cells already lining the mural TE.

The relationship between these three types of endoderm and their differentiation states is not fully known.Earlier work [30] suggested that the fate of PrE cells depended on the time when they were first generated from the ICM.The use of immunosurgery indicated that isolated ICMs produced a wave of PrE cells round their outer margin,if this first layer of cells is removed using a second immunosurgery treatment another layer of PrE is produced by the remaining ICM cells.The cells generated from the primary wave of cells delaminated from these isolated ICMs seemed to be committed to PE,while those from the secondary wave were apparently only capable of producing VE.This conclusion was mostly dependent on the observation that those PrE cells first derived from the ICM appear to form PE,but this could also be explained by a purely mechanical mechanism in which the first formed PrE cells became pushed round the blastocoel cavity by those PrE cells forming beneath and are therefore destined to become PE because of their juxtaposition with the mural TE.This model of separate fates for temporally different PrE cells,although attractive in it's simplicity,does not allow for either the interconversion of VE to PE or for the regeneration of PE cells from PrE once the VE has been formed and has been largely superceded by more flexible hypotheses of endoderm differentiation.

Other experiments involving tracing the fate of single ICM cells injected into genetically dissimilar host blastocysts [45] have indicated that both PE and VE cell types can be derived from a single ICM cell,thus suggesting a common lineage.It was therefore proposed that all PrE cells are capable of expressing both PE and VE cell types so that their phenotype is determined by the position in which they find themselves. There are two main hypotheses relating to their

interaction [61].The first,more traditional theory is that the fully differentiated cell types PE and VE are irreversibly derived from the bipotential precursor cell type PrE.A more interesting idea is that the differentiative state of these cells is not completely separated,so that PE and VE are transmutable and can differentiate from one endoderm type to the other,either with or without the intermediate stage of the PrE cell type.

To support this latter theory Hogan & Tilly have shown that VEX derived from a 6.5 day embryo will differentiate into PE if cultured in contact with extra embryonic endoderm undergoing transition into mural TE. Therefore,as with the earlier cells of the embryo,these differentiations do not seem to be completely irreversible and are probably to a large extent dictated by cell position and environment[61].

1.3.5. Cell Specialisation and Differentiation after Implantation- Mesoderm Differentiation and Germ Line Formation

The Visceral Endoderm is made up of two cell populations,the Visceral embryonic endoderm (VEE) which surrounds the embryonic ectoderm and the Visceral extraembryonic endoderm (VEX) which is adjacent to extraembryonic ectoderm.It has been shown [61] that the synthesis of AFP,which normally is produced by the 7-8 day stage VEE but not by the VEX can be induced in VEX cells within 12 hours and maintained for at least 48 hours if they are isolated from the Extraembryonic ectoderm.Embryo reconstruction experiments have shown that both VEE and VEX cells will synthesise AFP if placed adjacent to Embryonic ectoderm or in isolation,but not if in contact with extraembryonic ectoderm,therefore it would seem that the synthesis of AFP is controlled by a mechanism of cell-cell interaction and that the extraembryonic ectoderm directly inhibits the synthesis of AFP in its adjacent VE cells.

PE cells also show further evidence of differentiation at the post- implantation stages.From 8.5 to 13.5 days ,PE cells develop the Intermediate filaments (IF) vimentin as well as Cytokeratin which it expresses in common with most other embryo cells at this stage.The appearance of Vimentin is thought to be specifically related to reduction of cell-cell contact and to the independant existence of these cells following their detachment from the epithelial sheet from which they are derived [86].

The developmental fate of the embryonic Ectoderm in the posterior part of the embryo is to form mesoderm,both embryonic and extraembryonic.This has been demonstrated by experiments in which tissue from an 8 day embryo incubated in ^{3}H-Uridine was transplanted to a donor embryo and the fate of the labelled cells followed by autoradiography after 36 hours culture.Heterotopic injection of embryonic ectoderm tissue indicated that posterior and distal derived cells readily conform to colonisation patterns dictated by their new location,but that cells from anterior derived embryonic ectoderm showed some preference for definitive ectoderm differentiation rather than conforming to the position in which it was placed,although this did not indicate a built in mosaicism in embryonic ectoderm dictating their fate.These results show that the influence of position is even at this stage a major controlling factor in the development and fate of embryo cells.

Germ cells can occasionally be found,in the mouse embryo, in ectopic sites such as the adrenal gland.In 12.5-13 day embryos ectopically placed germ cells have all the morphological characteristics typical of a primordial germ cell.By the 14-15 day all ectopic germ cells have developed into oogonia,regardless of whether they are hosted in a male or female foetus.These oogonia then continue to develop and from day 17 to term all ectopic germinal elements enter meiotic prophase,reach diplotene and differentiate into oocytes with perfect adherence to mouse ovarian timetables.In post-natal animals,both male and female,these ectopic oocytes continue to mature and develop the ZP as though in their ovarian follicles,developing large antral follicles.However,by the 3rd week of life all these ectopic germinal elements disappear.These experiments show that mammalian germ cells can develop outside the gonads,but that the gonads of the male at least must exert some developmental environmental control.Outside the gonads all germ cells develop into the female form irrespective of their genetic sex [191],therefore indicating a certain degree of autonomy of the germ cell even at this early stage.

1.4. Teratomas and Teratocarcinomas

Teratocarcinomas are malignant tumours found naturally at high frequency in the gonads,and occasionally other sites,of certain strains of mice.They also occur at low frequency in most mammals,including human,and can be experimentally induced in a number of ways.

The major characteristic of teratocarcinomas is that they are formed of a variety of differentiated tissues and have a core of undifferentiated stem cells which are thought to generate the differentiated cell types.These undifferentiated cells are distributed randomly throughout the tumour in the form of small groups of cells.The benign form of the tumour,often called a teratoma,appears to consist entirely of differentiated cell types.Differentiation of a tumour is usually equated with its becoming benign as differentiated cells tend to stop division after a few rounds of mitosis and lose the tumorigenic properties displayed by the undifferentiated cells which have been shown to be embryonal carcinoma (ec) cells [1.5.1].

1.4.1. Teratomas and Teratocarcinomas

Mouse teratomas were first reported to occur at high incidence in strain 129 male mice by Stevens and Little [163] in 1954 and were then extensively studied both by Stevens and his co-workers [164] and by Pierce [125].There are certain important differences between those tumours derived from ovarian sources and those of testicular origin and therefore the two types are discussed separately.

Teratomas can be traced back as small nests of cells in the genital ridge of susceptible mice by the 15^{th} day of gestation in the male and are generally believed to be derived from the male primordial germ cell.Teratoma- like tumours have not been derived from female primordial germ cells.It has been suggested [99] that this is due to the fact that in the male foetus the germ cell remains diploid until after birth while the female germ cell enters meiosis on the 13^{th} day of gestation.

1.4.2. Testicular Teratomas

In 1954 Stevens and Little [163] found that about 1% of male mice of the inbred line 129 had these tumours congenitally.By selection of teratoma bearing lines this incidence had risen to 5% by 1969 [165].The tumour incidence in these mice was then further enhanced by the introduction of mutant alleles known to affect primordial germ cell development,from which teratomas were thought to be derived.Two of these genes W and Sl were separately introduced into 129 mice.In those 129 mice carrying the Sl mutation in the heterozygous

form (the homozygote embryo has no primordial germ cells and is lethal before the embryo becomes adult) the incidence of congenital teratomas rose to 10%.The addition of the W mutation had no effect on teratoma incidence,but a mutation (ter) which occurred during a backcrossing of W to 129 produced offspring with a dramatically raised,inheritable incidence.30% of the male progeny of this crossing had teratomas at birth and it is this strain (129/Sv-ter) which is now commonly used to generate spontaneous teratomas [164].

Evidence that teratomas are derived from primordial germ cells comes from th finding that neither genital ridge grafts of sl/sl embryos (where germ cells don't develop) nor grafts of genital ridges from pre-12^{th} day embryos (i.e. before the primordial cells have migated) do not produce teratomas.

The incidence of these tumours (in 129 mice) is the same for both pre- and postnatal mice,indicating that they originate in the foetus,probably at about 12 days (when the germ cells first migrate into the genital ridge) although they are not histologically identifiable until the 15^{th} day.Teratomas seem to be derived from some component of the genital ridge,as all teratomas of the 15-16 day old foetus are located in the seminiferous tubules which form a part of the genital ridge.Their ability to form a large number of differentiated tissues makes it seem likely that they are derived from primordial germ cells,which are pluripotent, rather than from sertoli cells which are not.

1.4.3. Ovarian Teratomas

About half of the female mice of the inbred strain LT/Sv have ovarian teratomas at 3 months of age [165].Ovarian teratomas originate from eggs that begin to develop parthenogenetically within the ovary,undergo an apparently normal cleavage stage and form blastocysts.At the egg cylinder stage (approximately 5 days) most of these embryos become disorganised and appear as clusters of undifferentiated embryonal cells and giant TE cells.

Like testicular teratomas,the ovarian tumours differentiate into a variety of tissues,the first to appear in both male and female tumours being neural tissue.However unlike testicular teratomas,ovarian teratomas are always associated with TE giant cells,possibly because they are derived from mature germ cells rather than from primordial germ cells although obviously environment may also have a very important role to play.

1.4.4. Experimental production of Teratomas

Teratomas can be induced experimentally in a number of ways.One of the first methods developed involves grafting of the genital ridge from 12 day embryos into ectopic sites in an adult recipient mouse.Genital ridges were explanted into adult mouse liver,spleen,kidney and testis.About 80% of 129 strain 12-day genital ridges transplanted under the adult testis capsule (a well vasculated site) develop into teratoma containing testes if they are transplanted under the testis of adult 129 mice.The timing is crucial as the number of teratomas induced decreases dramatically if genital ridges from foetuses older than 12 days are used,indicating that primordial germ cells undergo a maturation period in the testis at around 12 days after which they are resistant to teratocarcinogenesis.Teratomas do not develop from genital ridges from stages earlier that the 12^{th} day stage presumably because of the absence of the primordial germ cells.

The ability to produce teratomas in grafted genital ridges is dependant also on genotype.Some inbred strains of mice such as 129 and A/He give a high incidence of tumours in this way while other strains have intermediate,low or no susceptibiliy to this form of experimentally induced teratocarcinogenesis.

10% of ovulated LT/Sv eggs cleave in the ovary and then implant in the uterus.Shortly after implantation they then die.Teratomas have been induced from parthenogenetic blastocysts at 5 days.After implantation they were dissected out and transplanted into the testis of an adult syngeneic mouse where they developed into teratomas.

Similarly a high incidence of teratomas have been induced about 1 month after the grafting of 6-day embryos into the testes of adult mice.This indicates that these tumours are derived directly from the embryonic ectoderm of the embryo once the undetermined cell populations have become disorganised ,rather than from precursors of germ cells.EC-like cell lines (EK cells) can be also isolated directly *in vitro* from blastocyst stage embryos [1.5.2] and from parthenogenetic embryos [76] supporting the case for an embryo cell origin for the ovarian type of teratoma.Cleavage stage embryos grafted into testis or kidney yield a low incidence of teratomatous growths with many kinds of differentiated tissues and undifferentiated ec cells.

The embryonic development of the mouse is so rapid that by 8 days of gestation all cells have become determined and when embryos of this stage are grafted into the kidney or testis,all such cells differentiate.There are strain differences in the frequency with which teratocarcinomas can be produced by the grafting of embryos,C57Bl seem to be the most difficult while C3H embryos form teratomas readily.Site may also be important as more teratocarcinomas may be induced when embryos are implanted in the inguinal region rather than the flank [164].Preference for well vasculated sites such as under the kidney or testis capsules may have a more trivial explanation.Other determining factors may also be in operation to decide whether the tumour is malignant or benign,i.e whether or not it has a core of undifferentiated and proliferating ec cells.

Transplantable tumours have been established from both spontaneous and experimentally induced testicular teratomas and from embryo derived teratomas.Most are composed of many kinds of tissues in the early stages of transplantation but become restricted in potential with increased numbers of passages and they often stop growing because their stem cells have differentiated and therefore will no longer proliferate.If cells of a transplantable tumour are injected intraperitoneally they stimulate the peritoneal lining to secrete ascitic fluid and form small rounded groups of cells which have been called embryoid bodies because of their resemblance to 5- 6 day embryos.These structures were first observed within human testicular teratomas in 1939 by Peyron.Embryoid bodies generally have an inner core of epithelial embryonic ectodem like cells which are undifferentiated,and an outer layer of endoderm.Embryoid bodies of well established transplantable ascitic tumours are often quite uniform in size and some appear to be self regulating as they split into two when they attain a certain diameter.However not all ascitic tumours behave in this way and some cell lines form embryoid bodies which have a large range of sizes [165].

1.5. (*In Vitro*) and (*In Vivo*) Differentiation of Cultured Embryonal Carcinoma-Like Cell Lines

Malignant teratomas all have a core of undifferentiated "Stem" cells [1.4] which are capable of generating further differentiated tumours if transplanted.These cells are commonly known as embryonal carcinoma cells (ec cells).Single ec

cells derived from transplantable testicular teratomas (1964) and embryo derived teratomas (1974) have been grafted subcutaneously into host mice and have been shown to produce solid tumours composed of a variety of tissue,thus demonstrating the pluripotency of these ec cells.Pluripotency has also been demonstrated by several people who have made live mouse chimaeras using aggregated and injected blastocysts and ec cells,this is described in detail in a later section [1.5.4].

1.5.1. Embryonal Carcinoma Cells

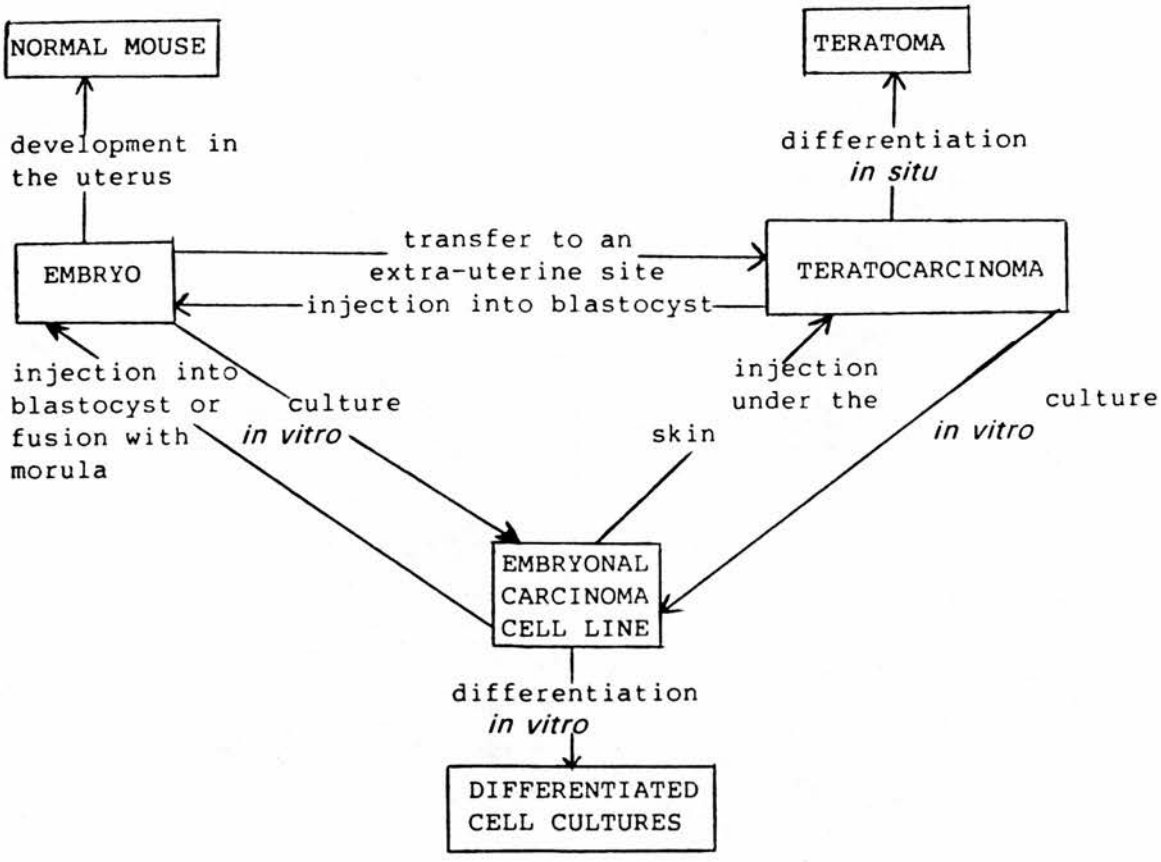
Permanent cell lines have been grown up from ec cells derived from tumours [163],and from blastocyst embryo implants into kidney and testes .More recently [37,5,99] ec cell-like lines have been isolated directly from the ICM of mouse blastocyst embryos.These last cell lines are known as EK [37] or ESC [99] cells and are discussed separately in section 1.5.2.

EC cells are relatively small,measuring 12-14 µm in diameter.Ultrastructurally they exhibit the characteristics of undifferentiated embryonic cells (ICM)[1.2.4].The nucleus is large and contains mostly euchromatin,with the nucleolus (2 or more depending mainly on the ploidy of the cell line) usually prominent [158].The narrow rim of cytoplasm around the nucleus is devoid of organelles except for a few mitochondria.A large number of free ribosomes are also usually present.This morphology is retained in tissue culture as well as *in vivo*,and is the same for all ec cells irrespective of whether they are of testicular,ovarian or embryo origin.

The establishment of cell lines derived from such undifferentiated cells into permanent stocks of cells that can be grown *in vitro* in large numbers has marked an important step in the history of developmental biology.This is because the ability to grow large quantites of development-related cells *in vitro* has made the study of development both more accessible to experimental manipulation and also allows the production of large amounts of homogeneous material for use in biochemical and immunological studies [46].Study of early development is severely limited in experiments using animal embryos,first because of the small size and number of cells at the early preimplantation stages [1.2.] and later because of the inaccessiblilty of embryos after implantation.It must not be forgotten,however,that ec cells are of a tumour

Figures 1:3 Interconversions of Mouse Embryonal and

Mouse Teratocarcinoma Cells


NORMAL MOUSE
development in the uterus
TERATOMA
differentiation in situ
EMBRYO
transfer to an extra-uterine site
injection into blastocyst
TERATOCARCINOMA
injection into blastocyst or fusion with morula
culture in vitro
injection under the skin
culture in vitro
EMBRYONAL CARCINOMA CELL LINE
differentiation in vitro
DIFFERENTIATED CELL CULTURES

origin and therefore may not entirely reflect the "normal" situation.

One of the major features of ec cells is the ability of most of them to differentiate both *in vitro* and *in vivo*,this property is often coupled with a dependence for *in vitro* growth on other cells.The requirement for these auxiliary cells,called feeder cells,has been overcome in some ec cell lines but this has in most cases been at the expense of the property of spontaneous differentiation.Such feeder independent cell lines can often be induced differentiate with restricted potency by agents such as retinoic acid.

To demonstrate the relationship between normal embryo cells and those derived from teratomas [Figure 1:3] ec cells have been incorporated into mouse embryos and live chimaeric mice have been produced,this is discussed in detail in section [1.5.4]

1.5.2. Derivation of EC-Like Cells from Mouse Embryo Blastocysts (EK Cells)

As described in the previous section,embryonal carcinoma cells have proved to be of great value as an *in vitro* model for early development.However ,the analogy of these cells with those of the early embryo inner cell mass must be regarded with some caution for several reasons.Not least of these reasons is that until recently all of the methods employed in the isolation of ec-like cells involved these cells in an abnormal,tumourigenic phase of growth.These processes may cause permanent changes in the properties of the resultant cells and thereby prejudice the results obtained from experiments in which they are used.For this reason workers have long been attempting to isolate continuous cell lines with embryo cell properties by direct *in vitro* isolation of cells from the mouse embryo.

Several people have now sucessfully established cell lines directly from mouse blastocyst embryos or ICMs (5,37,38,99 and 6) without the intermediate tumourigenic stage and it is hoped that these cells will behave,both *in vitro* and *in vivo*,in ways more closely analogous to the normal embryo.

The isolation of ec-like cell lines from mouse blastocysts was first reported by Evans et al [37] who gave these cells the name EK cells.Their method used a combination of ovariectomy and Depo Provera injection which induces diapause

and thus causes implantational delay of the blastocyst.This procedure allows the ICM to enlarge and increase in cell number.Low cell numbers were thought to be a cause of previous failures to isolate ec-like lines from mouse ICM cells.Therefore this process was thought to improve the chances of *in vitro* cell survival and so allow isolation of cell lines from these cells.Using this method Evans and Kaufman sucessfully isolated cell lines with a karyotype of 40XY which,by trypsin banding methods,appeared to be normal when compared to the karyotype of other somatic mouse cells.

However,it was found that,with improved culture conditions that this delayed implantation stage was unnecessary and both Martin [99] and later Evans [80] reported the establishment of pluripotent lines directly from normal mouse blastocysts.Martin used a combination of immunosurgery and ec cell conditioned medium to isolate cell lines which she called esc (embryonal stem cells).The immunosurgery technique was employed to remove the TE cells from the expanded blastocyst after removal of the ZP by pronase digestion.Immunosurgery has been previously shown [55] to give a high degree of separation of TE from ICM leaving the ICM cells virtually intact and viable.Isolated ICMs were then grown up into permanent cell lines in medium which had been previously incubated with a pluripotential ec line (PSA1).This medium may have contained a growth factor produced by these cells and released into the medium [99].

Evans (80) used medium enriched with B-mercaptoethanol and 20% foetal calf serum (FCS) to isolate cell lines from whole blastocyst embryos.As with earlier experiments which used implantational delay,embryos were cultured *in vitro* up to the egg cylinder stage and then when the TE cells had spread out across the dish away from the central clump of cells,this group of ICM derived cells was picked off and transferred to a fresh dish thus separating the TE cells from the rest of the embryo.

Later Axelrod et al [75] simplified this process by isolating ec-like cell lines from whole blastocyst embryo outgrowths grown in medium containing only 10% FCS in small (10µl) quantities and placed in small (terasaki) cloning wells.This procedure apparently enables the ICM cells to condition the medium in which they are being cultured sufficiently to support their growth into large enough numbers to allow the formation of a permanent line.

All of these methods so far reported require the use of STO "feeder" cells whose presence is apparently necessary to support growth at the early stages probably by preventing differentiation.

EK cells have also been isolated from parthenogenetic embryos [76] and their derivation may be influenced to some extent both by gender and genetic factors in that embryos from some mouse strains form EK cells more readily than do others [159].

By definition EK/esc cells closely resemble ec cells and ICM cells in their morphology and behaviour and it has now been shown that they also are able to form chimaeras [1.5.4] by blastocyst injection at high rates [17]. Some of the chimaeric mice obtained from such experiments have been found to contain EK cell derived germ cells although so far all of these animals have been male.If female germ line chimaeras were also to be obtained it would make it possible to obtain live mice composed entirely of EK cell derived genetic material.Obtaining normal live mice in this way would demonstrate the true totipotency of EK cells.

1.5.3. Differentiation of Embryonal Carcinoma-like Cells

EC cells can differentiate into a wide variety of cell types representing derivatives from all three embryonic germ cell layers and in some cases into embryonic tissues such as TE and parietal yolk sac [100].This has been observed both *in vivo* in the form of differentiating teratocarcinomas and *in vitro* in the form of embryoid bodies both in suspension and outgrowth form.

Many ec cell lines are capable of spontaneous *in vitro* differentiation and require to be cultured on a fibroblast feeder layer to retain their undifferentiated phenotype.Some ec cell types however have a reduced differentiative potential and differentiate spontaneously only at a very low rate such cell lines have often been termed "nullipotent" to distinguish them from highly differentiating lines such as PSA4.

However many of these "nullipotent" lines (such as F9,PC13 and R5/3) can be induced to differentiate at high frequency by treatment with various chemical inducers,the most highly studied of these are the retinoids and in particular retinoic acid (RA) which stimulates the production of endoderm in F9 and PC13

(the parent of R5/3) [61] and can also lead to differentiation which is predominantly of neuroglial and neuroectodermal origin.Other differentiation inducing chemicals include DMSO (which in certain cell lines stimulates differentiation into muscle) and HMBA which causes differentiation of some cell lines which do not respond to RA as well some of those which do.

Retinoic acid has been shown to have many other effects on developing cells and can interfere with the proximo-distal pattern formation in limb formation of the chick and the axolotl [94].RA has also been shown [111] to alter the distribution of cell-associated fibronectin in cultured sarcoma cells and to increase the glycosylation of specific cellular and cell surface glycoproteins and to decrease the production of secreted glycoprotein and glycosaminoglycans.

Retinoids have been shown in several studies (both *in vitro* and *in vivo*) to be capable of reducing tumour growth of ec cells [160] and other tumour types by causing them to differentiate and by dose-dependently inhibiting [59] the soft agar growth of both human and murine melanoma tumour cells *in vitro*.Retinoic acid has also been shown [75] to reduce the cell- cell adhesiveness in BHK21/C13 cells which normally grow in piled up aggregates and to increase the cell-substratum adhesiveness.Both of these features and soft agar colony formation are often associated with tumourigenicity although the correlation is imperfect.

In contrast to their action on many other cells retinoids inhibit both the differentiation and proliferation (in a specific and non-cytotoxic way) of normal human bone marrow cells [18].Differentiation of these cells (haematopoiesis) is stimulated in these cells by a variety of chemical inducers including both DMSO and PEG.

EC cell lines which can undergo metabolic cooperation (mec+) see [1.7] have been shown to lose this capacity in the presence of retinoic acid.Mutant lines have been isolated [153] which can overcome this block and cooperate normally in the presence of retinoic acid.It is not known whether this function of RA is related to its association with differentiation.

Pluripotent cells form endoderm before they differentiate into more complex cell types whereas "nullipotent"cells do not go through this stage before differentiation [100].This characteristic formation of an endoderm margin before further differentiation suggests that pluripotent mouse ec cells most closely

resemble day 4 ICM cells of the mouse embryo.

The detection of differentiation in ec cells is largely dependent on the observation of morphological changes which are apparently irreversible.However it is not always easy to unequivocably determine cellular differentiation by this method alone and several other tests of differentiation have commonly also been used.These methods in general exploit the switching on or off of genes (usually related to enzyme production) which have been shown to be characteristic of differentiation.Both specific markers (indicating one differentiated cell type) and non-specific markers (which simply indicate that differentiation has occurred) are available.A common marker of cellular differentiation is plasminogen activator which is produced in large amounts by a majority of differentiated cell types but is not expressed in detectable amounts by undifferentiated cell types such as ec cells.

Plasminogen activator (PA) is also produced in response to uv light [114] in cells with a DNA repair defect.It is therefore likely to be caused by unrepaired DNA damage and may represent a eukaryotic SOS function.It has been suggested that this SOS function may have a role in carcinogenesis as it is a highly specific serine protease closely associated with cellular transformation,neoplasia and tumour promotion.PA can be induced in several kinds of vertebrate cells by physical and chemical agents which cause DNA damage.Retinoic acid may also cause DNA damage and for this reason caution must be exercised when using detectable plasminogen acivator as evidence for differentiation.

1.5.4. Chimaera Formation

For some time it has been possible to create animal chimaeras by the aggregation of morula embryos *in vitro* and then placing the aggregates back into the uterus of another animal for gestation.This technique means that in theory mutant genes can be introduced into early developmental stages and their state and behaviour monitored as the embryo grows.It was recognised early on that this method could also be exploited to determine the developmental capacity of the embryonal carcinoma cell [1.5.2] and it has also been used to investigate the potential of EK cells [1.5.3].

However ec cell lines have been shown to have different affinities for ICM [118]

so that cells of the line PSA1-NG2 have a high affinity and are always found associated with ICM cells while PCC7-5 cells tend to be found outside the ICM cells and in contact with the TE.This affinity is a critical factor in determining the ability of ec cells to form chimaeras in aggregates and may be related to the ability of particular cell lines to form different tissue types.PSA1-NG2 (which forms aggregation chimaeras readily) differentiates easily into extraembryonic endodermal derivatives and to a lesser extent into TE.

The technique of blastocyst injection has been developed because it seems to give better results both with ec cells and with interspecific chimaeras.This method involves the microinjection of a single cell or small group of cells into the blastocoel of the early blastocyst embryo [1.2.3].This technique therefore overcomes the problem of TE affinity (ec cells) and uterine rejection [140,141] in interspecific chimaeras [see later on in this section].

One major problem in the use of chimaeras is following the fate of the different component cells.Until recently this was done fairly crudely by electrophoresis methods to measure isoenzymes such as Gpi which were known to be different in the two strains involved.The resolution of this method is limited by the skill of dissection out of the various embryo parts and inevitably involves the destruction of a large part of the embryo.This method of fate mapping therefore does not allow the pattern of mosaicism to be examined at the cellular level.

The use of interspecific chimaeras between Mus Musculus and Mus Caroli made by both blastocyst injection and aggregation [140] has enabled the development of a DNA probe [151] which recognises the satellite DNA of Mus Musculus chromosomes but not that of M.Caroli.This has enabled the development of an in situ hybridisation technique using the radioactively labelled probe in which the two cell types can be distinguished in mitotic spreads [139] and also in tissue sections .

It has been shown [42] that the developmental potential of ec cells (of the lines NG-2,PSA-1 and LT1-2D) after aggregation with cleavage stage embryos is by no means normal.Many foetuses examined at mid-gestation were morphologically abnormal with severity of abnormality correlated to the extent of ec cell contribution as indicated by Gpi analysis.Control aggregations of normal embryo cells produced no such abnormal foetuses suggesting that this

result is not due to the experimental procedure or aggregation.It is not known whether these findings are a result of the karyotypic abnormalities of the cell lines used or whether this limitation in developmental potential is characteristic of all ec lines.Other reports of mouse embryo and ec cell chimaeras have largely used the technique of blastocyst injection and apparently normal mice with substantial ec contribution have been obtained but still at fairly low frequencies.Germ line chimaeras have also been produced from certain ec cells.Stewart,T.A. [186] has obtained successive generations of mice with extensive contributions of ec derived tissue (often over 50%) by the injection of an established euploid ec line (METT-1) derived from 129 mice by injection into a C57Bl blastocyst.

1.6. Somatic Cell Hybrids -Their Isolation,their History and their Uses

A somatic cell hybrid is a cell which has been derived from the fusion of two genetically different somatic cells and which is capable of normal mitotic division and growth.The production of such hybrids from mammalian cells has been possible for some 20 years mediated by an external agent such as inactivated Sendai virus (a paromyxovirus) first used as a fusing agent for the hybridisation of Ehrlich's tumour cells [26] and of mouse and human cells [111].The virus was inactivated using u/v light or B-propiolactone although it is not thought that the DNA of the virus is incorporated into the hybrid cells.

Since 1975 [128] chemical fusogens have been available,the most common of these being polyethylene glycol.Several chemicals have been shown to have the property of causing somatic cell membrane fusion [78] and can be used either singly or in various synergistic combinations to cause cell-cell fusion.Although all methods of somatic cell fusion have their advantages and disadvantages,chemical cell fusion has the advantage of not introducing viral DNA into the cells which is of great importance in the genetic analysis of subsequent hybrids,especially when characteristics such as differentiation and tumourgenicity are being investigated,as these latter are known to be influenced by virally related DNA in the form of oncogenes.Therefore this section will concentrate primarily on methods of isolation of hybrids using chemical fusogens and on reviewing work which has already been done using such hybrid lines.

1.6.1. Chemical Methods for Somatic Cell Fusion

The first chemical agent to be used as a fusogen of eukaryotic cells was polyethylene glycol (MW 6000) which was used on monolayer cultures both of avian and mammalian cells to produce cell hybrids.One problem with PEG is that it is effective only within a narrow concentration range and at the optimum concentration for fusion (50-55% PEG) the cytotoxic effects of the chemical start to become significant.PEG is also much less effective as a fusogen when the cells are in suspension rather than in monolayer culture.Modifications of the basic method were therefore aimed both at reducing the toxic effects of the fusogen and increasing its efficiency in suspension.

The original protocol by Pontecorvo [128] was modified in 1976 first by Pontecorvo and then by A.Hales [53] for cultures in suspension.The latter modification involved the use of 15% DMSO in conjunction with PEG 1000 (previous methods used PEG 6000) in the reduced concentration of 41.6%.This modification was however found to be useful only for cells in suspension as it produced an excess of multinucleate cells when used on monolayer cultures [92],but the reduced viscosity of the lower MW PEG 1000 compared to PEG 6000 was an advantage for suspension culture fusion.

The proportion of bi- and Multinucleate cells increases steeply over the first 2 hours and then reaches a maximum of 30-60% overnight [92].Fusion efficiency has been measured by Atsumi,T. [4] who subtracted the nucleus ratio (i.e.the number of nuclei in polykaryons divided by the total number of nuclei in all cells counted) of the control cells from that of the experimental (fusogen treated) cell population to give a value which he called the "Fusion Index".This can be expressed as the following equation:

$$FI = \frac{\text{No.of Nuclei in polykaryons (experimental)}}{\text{No.of Nuclei in all cells(experimental)}} - \frac{\text{No.of Nuclei in polykaryons (control)}}{\text{No.of nuclei in all cells (control)}}$$

The use of DMSO to enhance PEG mediated somatic cell fusion was demonstrated for monolayer cultures also in 1976 by Norwood et al (121) who

used a 50% PEG 6000 solution in Dulbeccos medium containing 10% DMSO .This method was used by Oshima et al (123) to create hybrids from a nullipotent and a pluripotent cell line in 1981.

The use of a Ca^{2+} free environment has been shown (147) to reduce the toxicity of PEG in monolayer fusion experiments,if the cells are fused in PEG 1000 dissolved in Ca^{2+} free medium and incubated in Ca^{2+} free medium for a short while after treatment.This protective effect is,however,much more marked if PEG supplied by "Baker" is used rather than that obtained from "Koch- Light" which is far less toxic than the former even under normal monolayer fusion conditions with Ca^{2+} present.Yield of hybrid colonies therefore was increased only 2-fold when Ca^{2+} free conditions were used in conjunction with Koch-Light PEG,in comparison with the 100-fold increase in yeild obtained when Ca^{2+} was omitted from the Baker PEG experiments.However the yield for Koch-Light PEG is 80-fold that obtained with the Baker PEG fusogen even in the presence of Ca^{2+}.

Although the most widely used chemical cell fusogen has been polyethylene glycol,it has been shown [79] that a large number of other chemicals,or combination of chemicals also have this property.These chemicals were screened by Klebe + Mancuso [79],using the fusion of cells with PEG 1000 as the standard control.They identified 118 membrane active agents,over 20 of which were shown to have nearly the same efficiency of cell fusion as PEG 1000.In addition to this study Klebe + Mancuso modified and simplified the protocol for PEG fusion of cells in monolayer culture by eliminating the serial dilution steps [see methods chapter,2.3].The fusogen (PEG 1000) was dissolved in 0.15M HEPES buffer in a ratio of 1:1 w/v and was sterilised by membrane filtration as it had been observed that autoclaving the fusogen increased its acidity and so both reduced its efficiency and made it more toxic [79].

1.6.2. Post-Fusion Viability of Multinucleate Cells

One of the major obstacles to the production of viable hybrid cells using a chemically mediated fusion procedure is the toxicity of the treatment.This can result in multinucleate cells (polykaryons) failing to divide and so not forming hybrid cells in which the nuclei have been fused by the process of mitosis.

There are a number of factors which can affect polykaryon viability and these

can be roughly divided into two categories.Those effects occuring before or during fusion treatment form one category and include fusogen toxicity,presence or absence of Ca^{2+} and other metal ions,whether the cells are in suspension or monolayer form and the duration time of the treatment procedure.The second category involves those factors which have an effect after fusogen treatment and includes the length of time before the cells are trypsinised,medium type and medium supplements used for incubation directly after treatment.These categories are obviously fairly arbitrary and may overlap to some extent,in that some factors may have an effect on both pre- and postfusion viability.

These special conditions often apply only to the period of time that the cell is a polykaryon and after the first mitotic division hybrid culture becomes more straightforward.It has been shown [188] that heterokaryons behave very differently from either their parent cells or from subsequent hybrids and this is further discussed in 1.6.4.

1.6.3. Methods for the Selection of Hybrid Cell Lines from Fusogen Treated Cell Monolayer

The most common method employed in the selection of hybrid lines has involved the use of drug resistant mutants.The earliest experiments selecting hybrid lines involved the use of purine and pyrimidine nucleoside analogs which became important at the same time because of their early use in cancer chemotherapy (116).Drug resistance soon became a problem in the use of these drugs and was found to be the result of mutations which caused the absence or diminuition of a phosphoribosyl transferase enzyme,in the case of purine analogs,and of a kinase enzyme in the case of pyrimidine analogs.

The enzymes involved include HPRT (HGPRT,Hypoxanthine-[Guanine] phosphoribosyl transferase) whose absence causes resistance to the Guanine analogs 6-thioguanine,8-Azaguanine and 6-Mercaptopurine;and TK (Thymidine kinase) whose absence causes resistance to the Thymidine analogs Trifluorothymidine,5- Bromodeoxyuridine and 5-Iodo-2 deoxyuridine.Other enzymes whose absence causes purine and pyrimidine resistance include APRT (Adenine phosphoribosyl transferase) causing the resistance of a cell to Adenine analogs,dCK (deoxycytidine kinase) causing resistance to Cytidine

analogs and OP (Orotate phosphoribosyl transferase) causing resistance to 5-fluorouracil which is an analog of Uracil.

The use in somatic cell genetics of such analogs in combination with the appropriate mutant cell lines allowed a wide variety of studies which have included experimental mutagenesis,DNA-mediated transformation,gene mapping,transport,somatic recombination and ge).

This combination of salvage pathway mutants with purine/pyrimidine analogs has many advantages.Both purine and pyrimidine nucleosides are relatively stable compounds and occur only at low concentration in serum (in which most mammalian cell lines are cultured) and they are not liberated by the breakdown of serum components.The salvage pathway loci of mammalian cells have a relatively high mutation rate in comparison to microorganisms,but this is not too high in either the forward or reverse directions to invalidate genetic investigations.In the case of the HPRT enzyme whose loss is,in many cases,selected for by azaguanine and thioguanine the gene is situated on the X chromosome (in mice and many other mammals,including human) so making it hemizygous and facilitating the isolation of a recessive mutation.

Disadvantages of this system are that,at low analog concentrations,it is possible to isolate resistant variants at very high frequency which are however unstable and rapidly revert to the wild type in the absence of any selective pressure,the precise mechanism of this phenomenon is not known but may be due to the presence of reduced amounts of the enzyme in these cells which gives them some selective advantage over the wild type cells but which does not confer complete resistance this may be caused by gene reduplication making the loci prone to DNA recombination and the phenotype unstable.Tremendous variation in mutation rate (up to four orders of magnitude) has been observed between different cell lines.This may be due to the number of copies of a gene present or may also be a result of the juxtaposition of structural genes with other genetic material causing the inhibition of these genes and suppression of the mutation.

Finally ,one property of many mammalian cells is metabolic cooperation [Section 1.7] whereby nucleosides and other small metabolites may be passed between cells in intimate contact thus causing the death of drug-resistant as well as drug-sensitive cells in the presence of the drug.This property has also

been utilised in somatic cell genetics selection techniques.

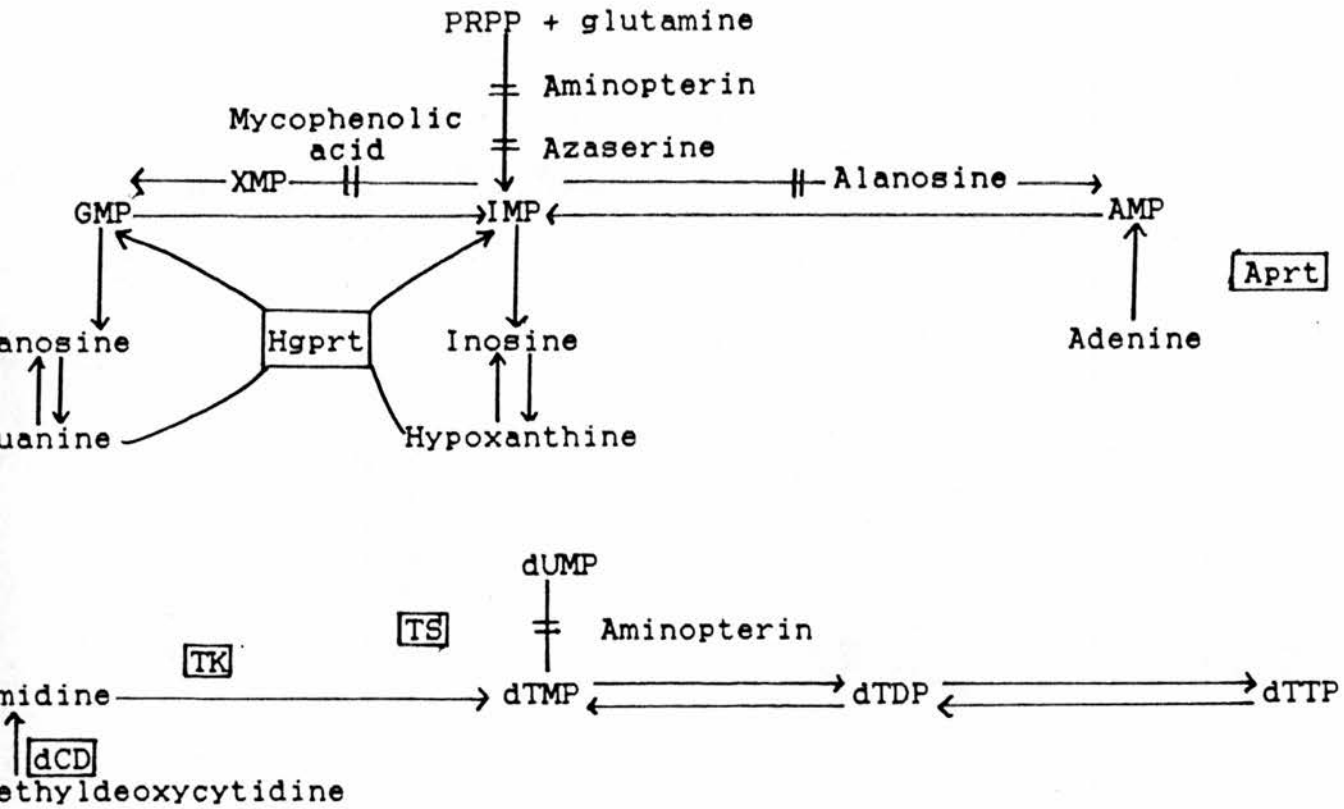
Mammalian cells are normally prototrophic for both purines and pyrimidines,therefore the addition of these compounds to culture medium is not essential to survival as the cells can synthesise them *de novo*However auxotrophy can be artificially induced by the introduction of folic acid analogs such as Aminopterin or Amethopterin (methotrexate) to the culture medium.These compounds are stoichiometric inhibitors of folic acid reductase and therefore,as well as other effects such as blocking the synthesis of certain amino acids,they can block both the endogenous purine synthesis pathway and the conversion of deoxyuridylic acid to Thymidylic acid in pyrimidine synthesis.This action makes the cell reliant on the presence of preformed precursor molecules,such as hypoxanthine and thymidine,to enable the synthesis of nucleic acid precursors through the alternative purine/pyrimidine salvage pathways using HPRT and TK enzymes respectively.This property has been utilised in reverse selection techniques and has led to the development of HAT medium [Figure 1:4] and an extension of the possibilities of the whole system,particularly the selection of hybrid lines.

The concept of utilising these mutants to facilitate hybrid selection technique was introduced by Littlefield (87) in 1964.This method caused the elimination of the parent lines which had different genetic deficiencies by a combination of selection techniques which allows hybrids with complementary functional genes to grow.Therefore Littlefield (87) fused two cell lines which were respectively resistant to 8-Azaguanine (and therefore deficient in HPRT) and 5-bromodeoxyuridine (and so deficient in TK) and cultured them in HAT medium.Neither of the parent cells would survive but hybrid lines had the complementary wild type genes and therefore could survive.This success with HAT selection has also encouraged the development of selective regimen for other drug resistant and for temperature sensitive mutants as well.

This basic selection method has been improved on and modified in the years since its inception.The selection can be made cleaner by growing the drug-resistant parents in the medium to which they are resistant for several generations before fusogen treatment to remove any revertant cells from the line.To allow recovery from the toxic block caused by drugs like Aminopterin ,hybrid lines selected in HAT medium can be cultured for several generations in medium containing Hypoxanthine and Thymidine (HT medium) before

Figure 1:4 The Biosynthetic Pathways of Purines and Pyrimidin

Shows the interconversions between compounds in both the pyrimidine and purine pathways and the various places in which these pathways can be blocked either by mutation (resulting in removal or inactivity of an enzyme) or by the introduction of drugs which specif- cally inhibit one or more conversions. These properties have been utilised in the development of selective media such as HAT (Hypoxanthine,Aminopterin and Thymidine containing) medium which can be used in somatic cell genetics techniques. Aminopterin and Amethopterin (Methotrexate) are analogs of Folic acid Reductase and stochiometrically inhibit the action of this enzyme.



rt Hypoxanthine-Guanine phosphoribosyl transferase
rt Adenine phosphoribosyl transferase
TK Thymidine kinase
CD Deoxycytidine deaminase

erence: Morrow,J. (1982) In/'Techniques in Somatic Cell
etics',edited by J.W.Shay.' 1-9.

transferring them to normal drug-free medium.

Many factors such as toxicity of aminopterin,serum purine concentration and cellular variation in nucleic acid metabolism,can influence the efficiency of HAT selection [26].Glycine is often an additional requirement for optimal growth when dialysed serum is used (THAG or HATG medium) because Aminopterin also blocks the synthesis of pyrimidines.Azaserine has been used in the place of folic acid reductase inhibitors to block endogenous purine synthesis alone in a further modification of HAT medium. Using the same principles as those employed in the use of HAT medium,selective media have been developed to isolate cell lines from cells using other drug resistant markers.These selective media include AA medium which selects for APRT+ cells using the antibiotic Alanosine to block the endogenous synthesis of AMP from IMP.Cells with the APRT enzyme can then make AMP from Adenine which is also added to the medium.

dCD+ (Deoxycytidine deaminase) cells are selected for in HAM medium containing Hypoxanthine,Aminopterin and 5-methyldeoxycytidine.Cells deficient in dCD die of Thymidine starvation as the enzyme is required for the conversion of Methyldeoxycytidine to Thymidine.This selection procedure therefore resembles HAT selection but uses dCD activity rather than TK activity.

Finally,a method for the selection of hybrids from HPRT- and APRT- parents uses GAMA medium which contains Azaserine to block endogenous purine synthesis and mycophenolic acid to block the conversion of IMP to XMP in the AMP to GMP pathway [Figure 1:4].Adenine and Guanine are then both required for cell survival which is only possible in the presence of both the HPRT and the APRT enzymes.

Other modifications to the HAT selection method involve the incorporation of other selective agents such as Ouabain,Polyene antibiotics and Diptheria toxin into HAT medium.The use of drugs such as Ouabain and Diptheria toxin was first introduced in methods developed for the isolation of intraspecific hybrids.Cells derived from rodents such as the mouse are approximately 10^4 times more resistant to the toxicity of these chemicals than are human cells. More recently cell lines of mouse origin have been isolated which are even more resistant to Ouabain (Oua^r) and this has enabled the use of this compound in conjunction with HAT medium to select hybrids which are both

Ouabain resistant and HPRT+.Such double mutant (HPRT-,Oua^r) cell lines are of particular value as markers in somatic cell hybrids as they can be regarded as "universal donor" lines and are useful for the fusion of one such marked line with a whole series of unmarked ones.

Similarly modified polyene antibiotics such as Nystatin methyl ester (NME) are differentially toxic to a variety of mammalian cells and so are also useful in the isolation of intraspecific hybrids.

1.6.4. The Uses and Behaviour of Somatic Cell Hybrid Lines

The production of hybrid cells with a single nucleus containing the combined genetic information of two separate cell lines is a very useful tool for the investigation of mammalian genetics without the necessity for long and elaborate breeding programmes,it also allows the possibility of investigating mechanisms which would otherwise be out of reach of conventional research methods.

Genome interaction can be studied in several types of experimental situation:in the heterokaryon,which is the immediate product of fusion;in proliferating hybrid cells retaining the essentially complete chromosome complements of both parents;in hybrid cells that have undergone segregation of chromosomes and in the products of the fusion of cell fragments (cybrids and reconstituted cells).

The study of such hybrids has revealed several interesting facts about genetic interactions.When two genomes are combined into a single nucleus (cell hybrid) the usual outcome seems to be purely additive so that recessive alleles are masked by the presence of their dominant counterparts and enzymatic mutants and other structural defects complement each other giving a pseudo-wild type phenotype.This has been shown to be the case for several loci whose genetics is known,for example a fusion of an HPRT- cell with an HRPT+ cell will yield a hybrid which produces the enzyme at approximately wild type levels [135,134,123],similarly the mutation G6PD- causing a lack of the enzyme G6PD (the gene for which is also located on the X- chromosome) is also found to be recessive and therefore not due to a dissociable inhibitor [134].

The use of somatic cell genetics in various studies has enabled the assignment

of specific genes to chromosomes an example of this is the enzyme Arginine succinate synthetase (ASS) the gene for which has been assigned to chromosome 9 [22] and was found to be a dominant trait.This enzyme is of importance because of its linkage to adenylate kinase,the nail patella syndrome and ABO blood groups. Previous indications of a non-random chromosome segregation of chromosomes 11,14,15 and 18 were confirmed.This occurs in the presence of any of the chromosomes from 1-10.

The non-random segregation of chromosomes is well documented in intraspecific hybrids and it is quite possible that this also occurs in interspecific hybrids also,for example 6-Tg resistance appears to be associated with non-random chromosome segregation in Syrian hamster cell hybrids [98].Many types of mammalian cell (human,mouse,chinese hamster ovary and several others) have been used in cell hybrid experiments with the specific aim of elucidating the genetic control of some function.The use of ec cells in somatic cell hybrids is discussed in the following section [1.6.5] and so will be merely mentioned in this section.

Other investigations using somatic cell hybrids include the finding that a deletion mutation on mouse chromosome 7 which blocks the production of several liver specific differentiated traits such as G 6-phosphatase expression is a regulatory gene and can be replaced by the corresponding gene from a rat cell in a mouse x rat hepatoma hybrid cell line.Using these hybrid lines it was shown that this deletion affected the production of at least 5 proteins without the elimination of their structural genes [27].

The study of the properties of heterokaryons has shown that they are functionally very different from either parental cells or from cell hybrids.Wright [187] has shown that heterokaryons formed from the fusion of mouse adrenal cells with differentiated chicken skeletal myocyte cells are capable of expressing both adrenal cell and muscle cell functions simultaneously.These cells are therefore mutually permissive for their differentiated functions and the mouse adrenal cells must therefore have an inducible myosin light chain gene as the mouse form of this protein was found in these heterokaryons.Control experiments in which adrenal or muscle cells were fused with fibroblast cells have shown that (in the heterokaryon) the fibroblasts can suppress muscle function but not adrenal function,this kind of experiment is important in the study of the genetic control of development.

When,in the late sixties,work was begun using somatic cell hybridisation to explore the kinds of regulatory mechanisms responsible for the acquistion and maintenance of the differentiated state [181] it was believed that the genetic analysis of the fusion products resulting from fusion of various kinds of differentiated cell would allow the deduction of the kinds of genetic mechanisms responsible for the expression of tissue specific genes.It has become clear now,however,that such an approach is oversimplified and that very complex processes may be involved which cannot be dissected using this method alone.

The majority of experiments using cell hybrids have been carried out on cells of permanent lines,many of which were derived from tumours,rather than on normal diploid cells.it is therefore difficult to tell whether the effect of a fusion event on the differentiated state of a cell is a function of its tumourigenicity or the result of a normal interaction of the genes which influence the differentiation capacity of a cell in the normal tissue of an animal.

So far it has proved difficult to establish pure cultures of normal mammalian cells which produce tissue specific protein in long term culture in the absence of modification of the karyotype.The use of tumour derived cell lines creates an additional confusing factor in the interpretation of results from somatic cell hybrids in terms of the genetics of cellular differentiation.This problem,however,although it indicates the necessity for caution in the extrapolation of results obtained from fusion products of tumourigenic cells to a more general model,does not invalidate the use of such cells to study differentiation and the results of several studies using hybrids from various types of differentiated and undifferentiated cells have yielded some important information and indications towards the understanding of the kinds of mechanisms which may be operating in cellular differentiation and have allowed the elimination of some kinds of models.In addition these studies have shown that there are fundamentally different mechanisms involved in the potential of cells to express genes and in the actual expression of these genes.

Control of differentiation is likely to be a complex process,Wright [187] has shown that heterokaryons made from the fusion of differentiated chick myoblasts to differentiation defective rat myoblasts produced detectable amounts of rat skeletal myosin chain indicating that the rat structural gene was still present and responsive to chick differentiative inducers.In the hybrid it was

found that an intermediate rate of differentiation occurred so that approximately 1% of hybrid myoblasts differentiated compared with 64% differentiation found in the chick myoblasts and 1/1000 th of this rate in the defective rat myoblasts.These results have been explained as demonstrating the need to achieve threshold levels of a secondary inducing molecules in response to differentiation stimulating conditions.This hypothesis explains many of the stochastic aspects of differentiation.

However the results of cell-cell fusions are,like genetics,not always so straightforward.The fusion of an AFP synthesising mouse hepatoma cell with an adult (rat) hepatocyte in which the synthesis of AFP is shut off yielded a hybrid in which there was active synthesis of AFP but in which rat AFP was not activated.The use of Southern blot techniques showed that both rat and mouse DNAs were present in their complete form.The co-existance of expressed and non-expressed AFP genes in these hybrid cells therefore suggests that their expression is dependent on a cis acting event [172].

The enhancement of the expression of cellular fibronectin (FN) in mouse-mouse somatic cell hybrids [142] has shown that hybridisation does not necessarily suppress expression of cellular FN and the production of much larger amounts of FN in these hybrids than is found in either of the parent lines suggests that the complementation of certain factors may operate in the hybrid to stimulate excess gene expression of FN.

The study of tumourigenesis using somatic cell hybrids has enabled the clarification of some of the mechanisms involved.Human cell hybrids from paired tumorigenic and non-tumourigenic HeLa fibroblast cells have shown that the protein tumour markers:the A-subunit of HCG and placental alkaline phosphatase are respectively correlated specifically with tumourigenicity and not correlated with tumourigenicity.Both were found in the hybrid cells but A-HCG was found only in tumorigenic cells and therefore is either involved in the process of tumorigenecity or is controlled by the same regulatory gene(s) [104].Because neoplasia is most likely a multistep process it would be useful to find whether either of these markers,or other markers,are present in any of the preneoplastic intermediate stages.

Similarly the study of the expression of common and private tumour specific cell surface antigens (TSSAs) has revealed that while the common TSSAs were

associated only with tumorigenic cells that the private TSSas were independent genetic traits and not related to the expression of the RSV (Rous Sarcoma Virus) src gene transformed tumour phenotype [83].

1.6.5. The Fusion of Teratocarcinoma Derived (EC

Cells,the Investigation of Differentiation and Cell Communication)

The use of embryonal carcinoma (ec) cells [1.5] in somatic cell hybrids has enabled the introduction of a new method for the investigation of the genetic control of development and of properties such as differentiation and cell communication which seem to be closely related to development.

Somatic cell hybrids between non-ec and ec cells are easily made [39] and have been used to study a variety of properties including the expression of the major histocompatibility complex (MHC) [11],X-chromosome inactivation [173],expression of haemoglobin [104] and metabolic cooperation [183,92].

Hybrid lines formed from two ec cells have proved more difficult to make (C.MacDonald pers.comm.) but have yielded some useful although apparently conflicting information on the genetic control of differentiation [123,135].In some teratoma hybrids retention of pluripotency has been observed,both in hybrids of pluripotent and nullipotent ec lines and also in hybrids between differentiated cell types such as diploid thymocytes [112].However other studies of hybrids between pluripotent and nullipotent ec cells [123] and also differentiated cell types (for example teratoma + Friend cell hybrids [83]) do not have the pluripotent phenotype of the ec cell type and are nullipotent (in the case of ec x ec hybrids) or differentiated (in the case of hybrids between ec cells and differentiated cell types).It is therefore only in the case of those hybrids between pluripotent lines and differentiated cell types where evidence has been obtained for the modification of determination ensuing from the hybridisation of somatic cells.This is complicated by the fact that not all such hybridisations result in this kind of modification.Some of these experiments are described in detail below.

Hybrid lines made from the fusion of a metabolic cooperation deficient (mec-) chinese hamster line (CHO) and mouse ec cells or mouse fibroblast cells have resulted in the complementation of the defect and the production of mec+ hybrid cells.Analysis of the chromosome loss in these hybrids suggests that

the mec function is located on chromosome 16 [183].The first demonstration that more than one locus may be involved in junctional communication came from the complementation of two mec- cell lines (R5/3 and LMTK-) which when combined as a hybrid formed a cell line which was mec+ and so capable of gap- junction mediated cooperation [92].This result indicates that the lesion causing cooperation deficiency in R5/3 is genetically distinct from the lesion causing cooperation deficiency in LMTK-.

The demonstration of the expression of class 1 MHC antigens in mouse ec x human hybrids at levels comparable to cells with a non-ec phenotype may help to elucidate the mechanisms whereby the embryonic genome programmes the expression of differentiated cellular functions.Expression may be in part due to cis control and is not found normally in ec cells,TE or in embryonic cells until after post-implantational development [11].

The non-expression of the HPRT enzyme and the presence of late or early replicating,presumably genetically inert DNA in the nucleus of somatic cell hybrids between an XO HPRT- mouse ec cell and and XY rat lymphocyte indicates that the X of the ec cell is inactivated.The morphological phenotype of all of these hybrids was characteristic of endoderm from the early embryo [173].

Experiments in which pluripotent ec cells have been fused with nullipotent ec cells or with differentiated non-ec cells have severally indicated that the nullipotent state is recessive [88,135],dominant [123] and multifactorial [108].

Oshima [123] has reported a decrease in the extent of In Vivo differentiation when a pluripotent ec line (PSA-1) is fused with an F9-derived (F9.22) "nullipotent" ec line.Hybrids were formed in 50% PEG/10% DMSO in Dulbeccos culture medium and were selected in HAT and Oua medium at a frequency of 10^{-3}.Hybrids were screened using flow microfluorimetry only to measure DNA content.5/8 isolated lines gave tumours composed entirely of ec cells while the remainder were composed of approximately 95% ec cells with a small focus of differentiated cells.*In vitro* less than 10% of the hybrid cells were found to secrete plasminogen activator after 6 days growth,this is comparable with F9 cells and indicates very little differentiation.Control "hybrids" (nullipotent x nullipotent and pluripotent x pluripotent) resembled the parent lines.

The opposite result to that found by Oshima [123] was obtained by

Rosenstraus et al [135] using cell lines which were apparently almost identical.They found that 9 independant hybrid clones formed from the fusion of the pluripotent line PSA-1 with a subclone of the nullipotent line F9 all displayed the same range of differentiated tissues in tumours as did the pluripotent parent line PSA-1 while the nullipotent x nullipotent control yielded ec tissue only.These hybrids were also isolated in HAT and Oua medium and were all found to be both HPRT+ and Oua resistant.Chromosome loss of 10-20% was experienced during early culture but over 50% of tumours formed within 4 weeks of injection and so were thought to be unlikely to be due to selection for a small sub-population of tumorigenic,differentiating cells.

littlefield and Felix [88] "rescued" terminally differentiating ec cells by their fusion to undifferentiated parent cells.The CBA derived ec line (H6) grows rapidly in suspension without Ca^{2+} but differentiates completely after approximately 6 passages in the presence of Ca^{2+} on gelatin if the cells are exposed to retinoic acid (RA).Under these differentiation inducing conditions the cells secrete plasminogen activator and produce large flat cells resembling endodermal cells.

Hybrid lines derived from the fusion of H6 cells with their differentiated derivatives yielded colonies which had the same undifferentiated phenotype as the parent ec line thus demonstrating that it is possible to "rescue" cells in the early stages of differentiation.Abortive colonies which also appeared in cultures of fusogen treated cells,but not controls,suggests that possibly some hybrid colonies retained the differentiated state and so did not survive.It has been suggested however that this may have been due to cross-feeding of parent cells in close proximity.

A series of hybrid lines made by McCue et al [108] by fusing cells which fail to differentiate in response to RA and/or HMBA has produced cell lines which have a variety of different phenotypes.Tumours made from these hybrid lines were scored for the presence or absence of various differentiated cell types as a measure of their differentiative ability.

Four classes of hybrids were generated,the first two were derived from the fusion of an RA- ,HMBA- mutant which did not have cRABP activivty (cRABP-) with either an HMBA- line which differentiates in RA and has cRABP activity (class 1) or with a line refractory to both RA and HMBA but which was

cRABP+.All of the hybrid clones derived in this way had cRABP activity and differentiated normally in response to both RA and HMBA.Tumours from these lines differentiated normally and in some cases to a greater extent than was found with the wild type cells from which the hybrid parent lines were derived.These results suggest that the mutations involved are all recessive and fall into different complementation groups.

The third class of hybrids was formed from the fusion of two different differentiation defective cRABP- lines and appeared to be at least partially complementary for responsiveness to HMBA and RA in *in vitro* differentiation.These hybrids had low,but detectable levels of cRABP but showed very restricted levels of differentiation *in vivo* with the majority cell type being ec.This result was not due to hybrid tetraploidy as control fusion experiments between cells of the same mutant lines did not differentiate either *in vitro* or *in vivo*neither did this last class of tumours have detectable levels of cRABP.

These results show that several different and unrelated genes are likely to be involved with the process of differentiation and indicate that the processes involved in cell culture differentiation (*in vitro*) may differ from those involved in tumour differentiation (*in vivo*)[108].However as yet there is insufficient evidence on the mechanisms involved in either kind of differentiation to determine whether or not these observed differences are of a fundamental or a trivial nature.

1.7. Metabolic Cooperation

Metabolic cooperation is a mechanism of cell-cell communication whereby cells can pass small metabolites (such as nucleotides) from one cell to the next via their gap junctions formed between closely apposed cells.This section describes these gap-junction s,their possible functions and experimental uses.Other forms of cellular communication are also discussed.

Much more is known about the ultrastructure and permeability properties of gap-junctions than is known about their physiological role(s).Gap-junctions appear to act as a molecular sieve allowing the passage of small molecules from cell to cell without contact with the extracellular medium.They are known to have both open and closed configurations and are likely to be also subjec to more subtle regulation [66].The discovery of tumour promotors and growth

factors has led to the speculation that the processes of cell growth and tumour formation may be mediated and regulated by intercellular communication via gap junctions.

1.7.1. Gap-Junctions and Gap-Junction Mediated Cell communication

The term "Metabolic Cooperation" was first coined by Subak-Sharpe,Burk and Pitts to describe the ability of variant tissue culture cells to incorporate a nucleic acid precursor when in the presence of wild type cells but not on their own [66].The process has also been called "contact feeding" and is distinguished from cross feeding where metabolites are transferred between cells via extracellular medium.Metabolic cooperation was redefined by Hooper and Subak-Sharpe in a review (1981) as the ability of cells to exchange molecules through permeable junctions formed at the sites of cell contact [67].They also review the techniques used to demonstrate metabolic cooperation and the evidence that these techniques share a common mechanistic basis,the properties of permeable junctions,factors controlling their formation and the possible functions of metabolic cooperation *in vivo*.

Animal cells contain several different kinds of junctions which form between closely apposed cells.These fall into three main classes which are desmosomes,tight junctions and gap junctions.Desmosome are regions of firm adhesion between cells and are associated with a cytoskeletal system which gives shape and rigidity to tissues.Tight junctions are found between epithelial cells and form gaskets which prevent the leaking out of luminal components.Gap junctions form channels of direct communication between cells [68] which are permeable to nucleotides but not RNA,DNA or protein.

In addition to these, some cells are capable of passing on molecules by means of cytoplasmic bridges.These structures are generally restricted to recently divided sister cells which have been shown to be capable of the passage of fluorescent dyes [148] and other larger molecules such as horse radish peroxidase [89].

Permeable gap-junctions are a common feature of most animal tissues and junctions with an apparently similar function,although different structure (plasmodesmata) have been found in plant tissues as well.Gap junctions are thought to consist of an array of hydrophilic pores [126] which regulate the

passage of substrates by means of pore size.These junctions allow the free exchange of small ions and molecules between all of the cells in coupled populations [127].Such limited syncytial interaction may contribute to the integration of individual cells into organised tissue.

Gap-junctions allow the passage of fairly low molecular weight molecules such as nucleotides,alkali metal ions and folic acid.This is likely to be determined at least in part,by the pore size of these junctions.However the cytoplasmic membrane appears to be impermeable to Calcium ions which on the basis of charge and size should be able to pass through gap-junctions and it seems that these ions have the facility of being capable of turning off gap junction function.Both Ca^{2+} and H^{+} ions are capable of reducing the conductance of gap-junctions [161].The sensitivity of junctions to H^{+} ions is,however approximately 1000 times greater than that to Ca^{2+} ions which do not have an effect at physiological levels.It would seem therefore that although pH probably plays an important role in the normal interaction of cells that calcium may only be important during cell death or after membrane disruption when the levels of calcium ions can rise to milli-molar levels.

The extracellular space of gap junctions are known to be permeated by the heavy metal salt lanthanum nitrate.This molecule can also enter the spaces in the gap junction if they are extracted from the cell.Both of these properties allow the use of Lanthanum nitrate to show up gap junctions in EM preparations [103].

Gap-junctions have a regular structure which is easily identifiable by electron microscopy.In sectioned preparations they appear as regions of close membrane apposition and in freeze fracture they are seen as areas of closely packed particles.They can be isolated and purified and contain 50% protein,7% cholesterol and 40% phospholipid.Finbow et al [40] have further refined methods for the isolation of the gap junction protein from cell monolayers or tissue homogenates using a purification method based on extraction with the detergent Triton-X-100.Using this method they have provided evidence that a 16K protein is involved in the structure of the gap junction protein although other workers have concluded that it is a 27K protein that is primarily involved in gap junction function.

Functionally,gap-junctions can be detected by their permeability properties

which include ionic coupling (measured electrophysiologically) which is recognised as an area of resistance which is several orders of magnitude lower than the ordinary resistance of of the cytoplasmic membrane,fluorescent dye transfer and nucleotide exchange [127].

Ionic coupling has been shown to play a role in the synchronisation of the passage of action potentials from cell to cell in primary cultures of myocardial cells thus causing a single combined contraction throughout the cells which are in close proximity [68].

1.7.2. The Isolation of Cells Defective in Metabolic Cooperation

The first indication that cells incapable of metabolic cooperation were available came from the observation that mouse L-cells,which do not appear to have gap junctions,do not cooperate with a wide variety of cells.L-cells have been widely used as negative controls [68] in a variety of techniques used to demonstrate metabolic cooperation.Other cell lines such as G3 which is a chinese hamster peritoneal cell line also show low intercellular communication with other cells [64].This property has however,been shown to be due to high homotypic specificity as these cells communicate at high probability with each other.

The initial demonstration that cooperation defective cell lines could be isolated in tissue culture was made by Wright et al [185,186,187] in 1976 who selected an apparently cooperation deficient cell line [mec-1A] from a polyoma transformed line of Syrian hamster cells.The selection procedure used involved the co-culture of TK- and TK+ variants of the same line in selective medium which was lethal both to the TK+ cells and to TK- cells which received nucleotide metabolites from the TK+ cells through their gap junctions.The selection procedure therefore allowed the preferential survival of TK- cells defective in metabolic cooperation.

However,as initial cell survival was quite high,numerous rounds of selection (46 in all) were necessary for the isolation of a line which was defective in cooperation and so,inevitably,several other changes also occurred which could be either secondary or directly related to the mec- defect.The line mec-1A was therefore different in size,morphology,growth rate and karyotype from the parent line.

The basis for the apparent loss of metabolic cooperation in this line of mec- cells was found to be due neither to a dilution of transferred nucleotides by the presence of enlarged endogenous pools of nucleotides nor to the failure of distinct internal pools to equilibriate [185].The deficiency can be reversed by treatment with dibutyryl-cyclic adenosine monophosphate (dBu-CAMP) and Theophylline which restores the ability to cooperate.

A comparison of the polypeptide profiles of the mec-1A line and its mec+ parent [187] has shown that there are at least 11 differences in the polypeptide content of the mec- cells in comparison to the mec+ cells.Six of these different polypeptides revert to the wild type (mec+) profile when the mec- cells are treated with dBu-CAMP and Theophylline which makes then phenotypically mec+ and so are obvious candidates for involvement in cooperation deficiency.In the Mec- cell line four of these six polypeptides displayed an increase in intensity in comparison to the wild type (mec+) line while two showed a reduction.

The second mec- cell line to be isolated in cell culture [153] was derived from the 6-Tg resistant ec cell line PC13TG8,via the line R2/1 which displayed intermediate behaviour between the mec+ parent line and the mec- line R5/3 in all aspects tested.Homotypic and heterotypic permeable junction formation between a mec- ec variant R5/3 and its parent PC13TG8 was studied by uridine transfer.The lesion was shown not to be due to modified specificity of cell recognition but to be caused by a deficiency in forming permeable junctions irrespective of contiguous cell type [64].

The isolation of this line was achieved after only 5 rounds of selection due to the increased efficiency of killing (survival was of the order of 10^{-5}) when using 6-Tg resulting in the accumulation of fewer secondary genetic changes.The karyotype of this line was however sub-tetraploid in comparison to the diploid chromosome count of the parent line and an increased resistance to 6-Tg was observed.In addition to these alterations it was found that the line has a decreased total gap-junction area per unit of cell volume and an increased surface area of microvilli per unit volume when compared to the parent line.The line was subsequently shown to be defective also in the transfer of alkali ions and of amino acids in addition to nucleotides [67] thus demonstrating that the R5/3 lesion reduces the capacity for the intercellular transfer of three unrelated categories of small molecule.This mec- phenotype

has been demonstrated both in heterotypic and in homotypic communication.

A small proportion of R5/3 cells were found to cooperate well both as recipients and as donors and this feature persisted even after the line was cloned.This has been explained [67] as being a result of the probability model of metabolic cooperation which states that a mec- phenotype is due to the reduction of the probability of communicaion channels forming between cells rather than a defect in the actual transfer mechanism.This would predict that the R5/3 lesion would have the effect of reducing the probability of R5/3 cells forming junctions with other cells but that once these were formed the transfer of metabolites should be normal.

A revertant line [65] which has restored capacity for metabolic cooperation has been isolated from the mec- line R5/3 by a "kiss of Life" method in which mec+,HPRT- cells are rescued from HAT toxicity.The properties of this line indicate that the increases in ploidy and 6-Tg resistance present in R5/3 which are retained by this revertant line (H2T12) are secondary and independent from the cooperation deficiency and the increase in microvillus density both of which disappear in the mec+ revertant.

The association of differences in gap junctions with a mec- line (R5/3) and the disappearance of these differences in the mec+ revertant (H2T12) and parent line (PC13Tg8) is strong evidence for a role of gap junctions in cooperation.The association of these events with an alteration in the cell surface microvillus density suggests that the cytoskeletal structure may be involved as it has been shown that surface microvilli play a role in the organisation of the cytoskeleton.

The R5/3 lesion has been shown to persist in endoderm-like cell derivatives [155] and so is not.likely to be a result of the differentiation state of the R5/3 cells.Tumours derived from R5/3 are almost entirely composed of ec cells.The inability to derive tumours from the H2T12 revertant line was resolved by cloning the parent line H2T (from which H2T12 was derived) and it was found that none of the tumours derived from these clonal lines contained any differentiated elements suggesting that the R5/3 cells had lost the ability to differentiate for reasons other than cooperation deficiency.

Selective communication between cultured animal cells was detected as selectivity in metabolic cooperation by Gaunt and Subak-Sharpe [49].The

majority of mec+ cell types examined (human skin fibroblasts,PC13,G3,Don,PyY) showed cooperation in almost all (95%) of their homotypic cell-cell contacts. This was not necessarily the case in heterotypic contacts,less than 10% of G3/Human fibroblasts and less than 30% of G3/PC13 contacts were observed to be positive for cooperation.On the other hand the "mec-" mouse L-cells formed permeable junctions in a greater proportion of heterotypic contacts than homotypic ones.This pattern was also found with PyY cells.

This selectivity of communication demonstrated in tissue culture cells could be of great importance in the whole animal.Gaunt and Papaioannou [48] have shown that mouse ec cells (PC13Tg8) will form permeable junctions with several different tissues of the early mouse embryo [1.2.3] including morula cells,ICM and endoderm,mesoderm and embryonic ectoderm of the 8^{th} day egg cylinder.In contrast TE of the mouse blastocyst and trophectodermal derivatives of the 8^{th} day egg cylinder such as embryonic ectoderm and ectoplacental cone cells showed little or no metabolic cooperation with PC13Tg8.

Intercellular communication through gap-junctions begins at the 8-cell stage in the mouse embryo [106] and coincides with the onset of compaction [1.2.3].Gap junctions were detected only in those embryos which were fully compacted although the use of cycloheximide to block protein synthesis indicated that the materials required for the assembly of these junctions is present by the late 4-cell stage.Lo and Gilula [89] have shown that until the late 8-cell stage when compaction takes place,communication between blastomeres is restricted to sister cells which are linked by cytoplasmic bridging.By the blastocyst stage the TE cells are linked to other TE cells and also to cells of the ICM.Immunosurgically isolated ICMs were also observed to be fully linked with the use of dye transfer.

1.7.3. Phenotypic effects and the Genetics of Metabolic Cooperation

As has already been described in a previous section [1.6.5] the creation of somatic cell hybrids between cooperating and non-cooperating cell lines is a useful method of studying the genetics of mammalian cells. Use of the techniques has indicated that the lesion(s) involved in cooperation deficiency are recessive in nature and can be corrected to the wild type by the fusion of a mec- cell to a mec+ cell.In hybrids between L-cells and normal human

fibroblast cells the frequent loss of human chromosomes generates segregants that have lost the ability to cooperate and have the mec- (L- cell) phenotype,the majority of these cells lacked the capacity for both ionic coupling and dye transfer and had no detectable gap junctions,but a few were found to be capable of ionic coupling despite the lack of gap junctions and an inability for dye transfer.

In addition to this,complementation in a hybrid between two mec- cells has demonstrated [92] that at least two independent genes are involved in cooperation deficiency since intragenic complementation is rare and has not been demonstrated in eukaryote cells.One of these genes has been tentatively assigned to the human chromosome 11 using segregation of the L-cell defect in human-mouse hybrids [68].

Positively cooperating cells are able to incorporate radioactive nucleotides not only by direct contact with a donor cell in autoradiography experiments (primary cooperator) but also by contact with a primary cooperator receiving labelled nucleotides from a donor cell.This can often lead to a gradient of labelling density in cells leading away from the donor cell and through surrounding contacting recipient cells [67].This facility demonstrates that metabolic cooperation of radioactively labelled nucleotides is reciprocal in that any one cell can behave simultaneously as a donor and as a recipient cell.

The need for cell contact has been demonstrated in a number of ways.Metabolic cooperation has been shown not to occur when cells are fed with medium conditioned by wild type cells,or when a coverslip of variant cells and a coverslip of wild type cells are cultured in the same medium but not in contact [67].Cooperation is not evident when cells are plated at low density,nor when they are in suspension culture.

1.7.4. The Role of Cellular Communication in Development and Tumour Formation and Differentiation

Gap-junctions may mediate the passage of growth controlling substances from cell to cell [68] and inhibition of this may be significant in the formation of some tumours.

Studies on the gap-junctional communication of cells in insects [180] have

revealed that,using intracellular injection of small fluorescent dye molecules,compartments are present across the wing disc epithelium of the Drosophila fly so that dye is readily transferred between the cells within the same compartment but not between cells of different compartments.The boundaries of the compartments appear to be defined by regions of poorly cooperating cells which are situated along the edges of the compartments.

The histiotypic specificity of metabolic cooperation can be investigated by the isolation of cells in different stages of differentiation using pluripotent ec cells [66].EC cells can also be of use in the development of antisera which can inhibit cooperation,compaction and differentiation in ec cells simultaneously.

HPRT- and HPRT+ clones isolated from the murine ec line PC13 form differentiated tumours on innoculation into syngenic mice,which have the same pattern of differentiation as that found in tumours formed from the parent line PC13 under the same conditions.Both of these lines are homotypically mec+ and have been shown to cooperate heterotypically with the CHO line Don and its derivatives.Both show little or no cooperation with derivatives of L-cells previously shown to be mec- [63].

A series of independent mec- lines which do not differentiate,have been isolated [155] using thioguanine kiss of death (4 lines) indicating a link between the two properties.All of these lines had a near diploid karyotype demonstrating that chromosomal duplication (as in R5/3) is not necessary for an ec cell to lose the ability to cooperate.

CHAPTER 2

METHODS

2.1. Materials and Sources

2.1.1. Cell Lines

With the exception of the STO mouse fibroblast line derived from SIM embryos,and the Human fibroblast line used in the mycoplasma test all other lines used were either of EC cell origin or were derived from cells of early mouse embryos.The characteristics and source of cell lines used,but not isolated during the course of this study are listed in Table 2:1.

2.1.2. Chemicals and Equipment

All standard laboratory reagents were obtained from Sigma(Fancy Road,Poole,Dorset) unless otherwise specified in this section.Pregnant mare serum Gonandotrophin (PMSG),Human chorionic gonadotrophin (HCG),Protease type iv (pronase),Napthol AS-MX phosphate,Nitroblue Tetrazolium (NBT),Phenazine Methosulphate (PMS),L-Malate,p-Nitrophenyl phosphate,2-amino-2,methyl propanol,Coomassie Brilliant Blue G-250,NAD,NADP,Mitomycin-C,B- mercaptoethanol,Dimethyl sulphoxide (DMSO) ,Ouabain ,Hypoxanthine ,Aminopterin ,thymidine and HEPES buffer were also all obtained from Sigma.

From BDH chemicals Ltd (Poole,England) were obtained Crude Glutaraldehyde (25% aqueous),Benzene, n-Hexane ,Ethanol ,Glacial acetic acid ,Methanol ,Trichloroacetic acid ,chromic acid ,Kenacid blue ,Leishmans ,Giemsa ,Gurrs buffer tablets (PH 6.8 and 7.2) Progesterone , silica gel Culture media and supplements (NEAAs,Gln,pyr,GMEM,) Phosphate buffered saline tablets,Ca 2+-free medium,were all obtained from either Gibco Europe Ltd (Trident House,PO Box 35,Renfrew road,Paisley,PA3 4EF) or Flow Laboratories ltd (PO Box 17,Second Avenue Industrial Estate,Irvine,Ayrshire,Scotland RA12 8NB) Penicillin,Streptomycin and Trypsin, were from Flow

Table 2:1

ORIGIN AND CHARACTERISTICS OF MAMMALIAN CELL LINES USED IN THE COURSE OF WORK REPORTED IN THIS THESIS

Shows the full name of the line,what it was used for, its relevant characteristics and where it was first derived from.

name	Type	Origin	Reference	Gel	Feeder dependence	Differentiation	Metabolic Cooperation	Karyotype	Genetic Markers	Remarks
STO	fibroblast	SIM embryos				-	+	55-60	Oua Hprt	used as feeder layer for ec cells
HFL	human fibroblasts			-	-					used in mycoplasma assay
scc-PSA4	mouse EC	from OTT-5568 tumour	Martin,G + Evans,M.J. (1975)PNAS 72 1441-1445	-	+	+	+	40 XO trisomy 3	Oua Hprt	parent line in hybrid selection
R5/30A	mouse EC	Oua derivative of R5/30A	Slack,C.+ Morgan,R.M.+ Hooper,M.L. (1978)	+	-	-	-	73 2 metacentrics	Oua Hprt	parent line in hybrid selection

Sera (foetal calf,newborn calf,chicken,) were obtained from Flow,Gibco,Sera-labs Ltd (Crawley Down,Sussex,RH10 4FF) or Northumbria Biologicals Ltd (South Nelson Industrial Estate,Cramlington,Northumberland,NE23 9HI) NuSerum (serum substitute) was from Collaborative Research.Plastic tissue culture ware was from Nunc (distributed by Gibco),Falcon (div.Becton Dickinson & co.,Cockeysville,MD 21030,USA) or Flow,"Optikon" cloning wells from Northumbria Biologicals,and "Thermanox" plastic coverslips were from Lux (obtainable through Flow).

PEG 1000 and PEG 6000 were both from Koch-Light laboratories LTD (Colnbrook,Bucks. England).Coomassie Brilliant Blue G-250 dye reagent concentrate was from Bio Rad laboratories (32^{nd} + Griffin,Richmond,California) .Bovine serum albumin (BSA) standard was from either Sigma or Bio Rad.

Radiochemicals 5-3H-uridine [110 mCi/mg], were from The Radiochemical group (Amersham,England).Photographic materials came either from Ilford LTD (Basildon,Essex) (Nuclear research emulsion K2 and 35mm PanF B/W film) or from Kodak-Pathe (EMB,71102 Chalon-sur-Saone,France)(D19 developer and Ektachrome colour slide film).

Meldola blue was from Boehringer-Mannheim GmBH (The Boehringer Corporation [London] Ltd,Bell Lane,Lewes,East Sussex BN7 1LG), and scintillation fluid from Packard.Guinea pig serum (complement) was obtained from the Scottish antibody production unit (SAPU,Glasgow and West of Scotland Blood Transfusion Service,Law Hospital,Carluke,ML8 5ES,Lanarkshire,Scotland).A-1 Foetoprotein was from Dako LTD (22,The Arcade,The Octagon,High Wycombe,Bucks.HP11 2HT).

2.1.3. Mice

For origins and characteristics of mice used see Table 2:2.Strain 129 mice were obtained from Dr Martin L.Hooper's stock,CBA and C57BL stock were bred from mice donated by Dr Roger Gosden,SEY and TCD57Bl from mice donated by Mrs Ruth Clayton,and the C57BL-Modl^{null} stock from mice obtained from Professor Richard L.Gardner.

All mice were maintained in the faculty of medicine animal area in George Square,Edinburgh and experiments were carried out in accordance with the

Table 2:2

ORIGIN AND CHARACTERISTICS OF MICE USED IN THIS STUDY

Lists the mice used in experiments in Chapter 7 and for *In Vivo* differentiation experiments in Chapters 3,4 and 5.

Name	Background	Where obtained	Genetic markers	Coat colour	Remarks
C57Bl	C57Bl	R.G.Gosden Dept.Physiology Edinburgh		Black	used in enzyme studies
C57Bl.Modl (null)	C57Bl	R.L.Gardner Dept.Pathology Oxford	Deficient in L-malic enzyme (Modl-null)	Black	Source of blastocysts in the isolation of Modl and other Modl.lines.
129	129	M.L.Hooper Dept.Pathology Edinburgh		White	Used to test tumourigenity of hybrid lines derived from 129 ec cells.
TcD57/Bl	C57Bl	R.Clayton Dept.Genetics Edinburgh	small eye (see note)	Black	Used as a source of blastocysts.

note:

i) the allele causing small eye in TcD57/Bl strains of mice is lethal in homozygous form and the stock were therefore maintained as heterozygotes (Sey+/Sey-).

requirements of the Cruelty to Animals Act(1876) under a home office licence (number Ela 23/8954/1) and certificates (A1 and B1) granted to myself on the 5th of April 1982.

2.2. General Tissue Culture Techniques

2.2.1. Washing Glassware

Glassware for use in tissue culture was washed and kept separately from other glassware to avoid contamination with toxic chemicals which could interfere with cell growth.Pipettes were soaked overnight in Calgonite (100g/l), washed in water and then in 0.01M HCl.They were then rinsed thoroughly in running tap water and once in distilled water and were then dried in a hot air oven at 180° C.

Glassware was sterilised by heating to 160° C for two hours,metal canisters and foil caps being used to maintain sterility,before use in tissue culture in still air or laminar flow hoods.

2.2.2. Culture Medium

Routine tissue culture was done using Complete Medium [appendix 1] supplemented with either newborn calf serum (NCS) or foetal calf serum (FCS),or occasionally NuSerum all at concentrations of 10% unless otherwise indicated.B-Mercaptoethanol (10^{-4}M) [123] was also added routinely to culture medium unless specifically indicated otherwise.

Serumless medium (CMX) was made by omitting the calf serum supplement from the complete medium recipe.EC10 and EC20 medium for culture of embryoid bodies were made up without Pyruvate, NEAAs, or B-Mercaptoethanol.

In certain circumstances,i.e for the isolation of hybrids or of embryo-derived cell lines,it was found helpful to use "enriched"medium [2.3.4] which was simply CM supplemented with 20%,rather than 10%,FCS.

Other special and selective media are described later on in this chapter and all

recipes and details of media are summarised in appendix 1.

2.2.3. Subculture and Growth Conditions

Cells were generally maintained in small plastic tissue culture bottles ($25cm^2$) incubated at 37° C and gassed with a mixture of 5% CO_2/95% air to allow pH equilibriation using a sterile plugged pipette and taking the gas from a cylinder by means of a rubber tube and an adjustable tap.Cells which were being cultured in dishes (which are not air tight) were incubated in a 37° C incubator (Labmark,or Forma Scientific) in which was maintained an atmosphere of 5% Co_2/95% Air.

Cells were subcultured when they formed a confluent monolayer,usually every 3-4 days.Most EC cell lines required to be fed at least once during that time and some required a medium change once per day.This was monitored by inspecting the monolayer daily using the phase contrast microscope and by observing the phenol red indicator incorporated into the culture medium which became yellow when the mixture was turning acidic and was therefore requiring a medium change.

Cell lines were subcultured when confluent;they were first washed twice with serum - free isotonic buffered saline (PBS,appendix I),which prevents serum interference with the trypsin and therefore facilitated the removal of cells from the plastic culture surface without damage.Cells were then disaggregated using TVP which is a mixture of Trypsin (0.25g/l),Disodium EDTA (0.37g/l) and chicken serum (10ml/l) in PBSA (appendix 1).This process usually required only a few minutes incubation at room temperature,although some of the feeder dependent cell lines which grew tightly clumped together were left in the enzyme mixture slightly longer and also put into the 37° C incubator for complete removal of cells.

Because of the extracellular matrix produced by the Modl cell line it was found that the use of trypsin in the form of TVP to subculture these cells caused the partial breakdown of the matrix into irregular "lumps" which caused difficulties in growing up subsequent monolayer cultures.Therefore it was found that these cells could be satisfactorily dissociated from their growing surface using EGTA (0.5mM) alone [167],and this was done routinely in all later experiments involving Modl cells.

When cells were successfully disaggregated by either TVP or EGTA ,the reaction was stopped by the addition of normal culture medium containing serum.The phase contrast microscope was used to check that a single cell suspension had been obtained.For stock subculture to a fresh $25cm^2$ bottle a split ratio of 1/10 - 1/20 was normally used although this depended,obviously,on the rate of growth of cells and when they were next required to be used.

When plating out cells at cloning density,usually in plastic dishes,cells were counted with the aid of a haemocytometer and the cell mixture was then diluted and 10^3 cells per dish (regardless of dish size) were added as accurately as possible.

2.2.4. Preparation of Feeder Layers and Gelatin Treatment of Tissue Culture Surfaces

For growth of some cell lines [Table 2:1) it was necessary to further treat the culture surface of plastic bottles and dishes to facilitate spreading,attachment and growth without differentiation.

Gelatin layers were prepared by treating the plastic culture surface with a sterile 0.1% solution of gelatin and allowing adsorption onto the plastic surface for 15-30 minutes at 4° C.The surplus liquid was then discarded and the culture vessels used as normal [12].

Feeder layers were prepared by incubating confluent layers of live STO cells with CM10 supplemented with 10µg/ml of mitomycin C.After two hours at 37° C the medium was removed and the mitomycin C washed off by two washes of CM10 medium [101].The STO cells were then washed in PBS and trypsinised as described in the previous section,and the cells were then counted out and plated into tissue culture dishes or bottles as required at a cell density of 4×10^4 cells/cm^2 (10^6 cells per $25cm^2$ bottle).These feeder layers could then be kept for up to a week,maintained in CM10 (NCS) at 37° C and 5% Co_2.To use the feeder layers,the maintenance medium was removed and cells plated out onto the attached cell layer and then fed with the appropriate medium.

Because Mitomycin-C has suspected carcinogenic properties,precautions were necessary in the handling of this material.These were similar to those

employed in the handling of radioactive chemicals.A spillage tray covered with tin foil was used in all manipulations and plastic gloves were worn at all times.Pipettes and other non-disposable equipment used in transfering all contaminated material were soaked overnight in Kirbychlor (in the case of mitomycin C) or decon (in the case of radiochemicals) before being washed in the normal way.

2.2.5. Mycoplasma Checks

Regular checks were performed on all cell lines in regular use and also on those isolated during the course of this project,because mycoplasma can cause important deviations of behaviour and is undetectable by normal phase microscopy and therefore can not be monitored by the microscope as were other bacterial and fungal contaminations.

There are several different methods used for mycoplasma detection,but the one used routinely in this laboratory was a modified version of that of [24] Chen,T.R.HFL cells were set up in 60mm dishes at a density of 10^5 cells per dish.Test cells were then prepared by scraping a $1cm^2$ area of cells into 2ml of their own supernatant and adding O.2ml of this to the dishes of HFL cells.After 24 hours the medium was changed and then after a further 4 days incubation the cells were fixed by the dropwise addition of 3ml methanol/acetic acid (in a 3:1 v/v ratio).After 1 minute this medium was replaced with a further 3ml of fixative and left for 5 minutes,after which cells were washed with fixative and allowed to dry.The preparations were then stained for 10 minutes with Hoechst 33258 (0.05µg/ml,in complete PBS),5ml per dish.Excess stain was then removed by washing the dishes twice in distilled water and a thin coverslip was applied with the aid of two drops of McIlvaine's buffer (11.37ml 0.2M Na_2H_{PO4},and 8.62ml 0.1M citric acid,pH 5.5) and the edges sealed with stencil correcting fluid.

Preparations were then examined at 1000 times magnification under the fluorescent microscope for the presence of mycoplasma in the cytoplasm of the HFL cells.

2.2.6. Long Term Storage of Cells

Stocks of cells were maintained in 0.5ml quantities in small vials frozen at -196° C in the vapour above liquid nitrogen.Stocks were prepared from large bottles of confluent cells which had been trypsinised.Medium consisting of CM10 plus 10% DMSO was prepared and cells were washed once in this mixture at room temperature in order to equilibriate them with the DMSO.The cells were then spun down in an MSE bench top centrifuge at 1000 g for 3 minutes to remove the CM10 + DMSO.The cells were then resuspended (at a density of approximately 10^6 cells/ml),in CM10 + DMSO(10%),0.5ml per vial and 10 vials per confluent large plastic bottle.

These vials were then frozen down slowly by leaving overnight in the - 70° C freezer in an insulated rack.They were then transferred the following day into the liquid nitrogen freezer.

To reverse this process and recover cells from frozen stock,a single vial was removed from the liquid nitrogen and thawed rapidly in the 37° C water bath.The cells were washed once in CM10 to remove the DMSO which is cytotoxic at room temperature and were then placed into small culture bottles as usual.The medium was changed after 24 hours in order to remove dead and non- attached cells.

2.2.7. Staining of Cells and Colonies

i) Leishmans Staining Unless otherwise indicated cells and colonies in experiments were routinely fixed and stained using a filtered solution of Leishmans stain (1.5g/l) in methanol.The staining solution was left on the cells for 5 minutes at room temperature to fix them,after which time an equal volume of tap water was added and the cells left for a further 5 minutes before the staining mixture was washed off and the dishes left to dry.

ii) H & E staining Paraffin sections were deparafinised in xylene and then hydrated through graded alcohols (100%-65%),they were then rinsed in water and stained with Alum Haemotoxylin (Harris') for 3 minutes,rinsed in water and then dipped in 1% acid alcohol for 3 seconds.The preparations were "blued up" in Scotts tap water substitute,rinsed in water and then stained for 20 seconds

with 1% aqueous Eosin (supplemented with 0.5% Phloxine and 0.5% $CaCl_2$,to enhance the brightness of the stain).The sections were then finally rinsed,dehydrated through alcohol,cleared in xylene and mounted in synthetic resin.

iii) PAS Staining Paraffin sections for PAS staining were deparaffinised and hydrated as described in 2.2.8.(ii) and then incubated in 1% periodic acid for 5 minutes,rinsed and incubated with Schiff's reagent for 10 minutes before counterstaining with Haemotoxylin,dehydrating,clearing with Xylene and mounting as usual.

2.2.8. Isolation of Clonal Lines

Cell lines were cloned by dilution of trypsinised cells in CM10 to a single cell suspension of less than 10 cells per ml.This cell suspension was then plated into 0.28 cm^2 cloning wells of volume approximately 0.35 mls which were either previously treated with gelatin or contained an STO feeder layer [section 2.2.4].Cells which were difficult to clone were plated out into gelatin and feeder layer treated "Optikon" cloning wells which had a surface area of about $1/3^{rd}$ of the 0.28 cm^2 wells and so allowed culture in very small volumes of medium (20-10 µl). Colonies were observed as they grew up and those with only one colony per well were chosen to be grown up as stock.Permanent stocks were obtained by the gradual transfer of these colonies into larger wells and finally,into small plastic bottles,as they became confluent.

2.2.9. Formation of Embryoid Bodies

Except where otherwise indicated,aggregates were prepared as described below.However,in some cases the length of time for which aggregates were allowed to incubate was varied at the suspension stage and,where appropriate,this has been individually recorded in the results section.

Since the object of this procedure was to enhance differentiation, EC10 medium [appendix i] was used as culture medium,unless EC20 was indicated.Cells were initially seeded at high density (2.5×10^6 cells/60mm dish) into tissue culture dishes and fed as necessary,usually about once per day,for 4-5 days with EC10 until piled-up aggregates had formed.These cell aggregates were then

detached by blowing 5ml of fresh medium across the surface of the dish.Then using a wide-bore pipette the aggregates were transferred to a conical-bottomed universal container.This procedure was repeated until all the aggregates had been removed.

After the cell aggregates had sunk to the bottom of the universal,the medium was changed and the cell aggregates plus medium were transferred to 90mm petri dishes.These were bacteriological dishes which had not been treated for tissue culture and therefore the majority of cells would not attach to the plastic surface but would remain suspended as cell aggregates.These suspended bodies were then incubated as usual for at least 6 days,feeding as required by transferring to conical universals and changing the medium once the embryoid bodies had sunk to the bottom of the universal.By 6 days the aggregates,with the exception of those of the Mod1 line,had developed a 2-layered appearance,and at this stage they were either allowed to develop further without interference or were plated out onto 60mm tissue culture dishes to allow formation of outgrowths.Alternatively they were fixed in Bouin's fluid,embedded in paraffin wax and sectioned (4-5μm) onto slides when they were then stained either using Mayer's Haematoxylin and eosin [section 2.2.8.ii] or,as in the case of some of the Mod1 EB slides, with PAS instead [section 2.2.8.iii] and observed under the light microscope.

2.3. Selection of Hybrid Lines

2.3.1. HAT and Ouabain Medium

Serumless medium containing Hypoxanthine (10^{-4}M),Aminopterin ($8x10^{-7}$M),and Thymidine ($2x10^{-5}$M) was made up using sterile stock solutions of each,made up to 100 times the required concentration.The Hypoxanthine had to be dissolved at alkaline pH but the Thymidine and Aminopterin were easily soluble at neutral pH and room temperature.Care was taken with the Aminopterin and was weighed out by a male member of staff since due to suspected teratogenic properties,it is not recommended that the solid is handled by women of childbearing age.

To make a saturated solution of Ouabain the HAT medium was added to Ouabain (0.219g/100ml),mixed well,and then made sterile using a 0.2μm

millipore filter,the final concentration of Ouabain being $3x10^{-3}$M.Serum (10-20% as specified by the experiment) was added after filtering to avoid the clogging up of the filter.

To prepare HAT only or Oua only medium the relevant parts of this procedure were followed,missing out either the HAT or Oua stages as required.When Oua is not added the filtering stage is also unnecessary and stock solutions can be added directly to CM(10-20).

2.3.2. Preparation of Fusion Mixtures

The fusion mixture was modified through several attempts at hybridisation,the successful preparation being that containing PEG 1000 and HEPES buffer.The methods are listed here in chronological order.

i) Serial dilutions of PEG 6000 with CM(X) [53,128] [49,121] were made as follows:50g of PEG 6000 was weighed out and autoclaved to make it molten and sterile.To this was added 50 ml of CM(X) giving a 1+1 (w/v) dilution of the PEG 6000.In later experiments the PEG was made molten by heating in a 60° C water bath and then made sterile by filtration through a 0.2µm millipore filter.This prevented the solution from becoming too acidic. Serial dilutions of 1+3,1+7,and 1+16 were made by further dilution with CM(X) of the 1+1 solution.The fusion mixture was made 2-3 days prior to use as this was reported to improve its fusion properties.

ii) PEG 6000 was diluted 1+1 in CM (10% DMSO),without serum, using filtration by 0.2µm millipore filter as the sterilisation method [121].

iii) PEG 1000 was melted in a 60° C water bath and then added to an equal volume of 0.15M HEPES buffer containing phenol red indicator,and adjusted to a pH of 7.55[79].The Solution was made sterile by filtration through a 0.2µm millipore filter.

2.3.3. Preparation of STO conditioned medium

STO conditioned medium was made by removing medium which had been incubated with STO feeder layers and filtering it for use in growing feeder "dependent" cell lines [154].

In the process of developing this technique several permutations of the basic method were used,these are discussed in the results [Chapter 3],and the final procedure only is described below.

Several large plastic bottles of STO feeder layers,$7x10^6$ cells per bottle, were prepared [see section 2.2.4,this chapter for method] and were incubated with CM10 for 7 days.The medium was then removed and pooled into one container when it was then filtered to remove cells and cell debris which may have been present using large size 0.2µm disposable millipore filters.

This filtrate was then supplemented with glutamine (1ml of stock solution per 100ml of medium),B-mercaptoethanol (10^{-4}M),and 5-10% additional serum.

2.3.4. Preparation of Enriched Selective Medium

Where enriched medium is indicated CM supplemented with 10^{-4}M B-mercaptoethanol and 20% FCS was used.The development of this method is discussed in the results [chapter 3,section 3.1.6].

In some cases medium was previously incubated with STO feeder as described in the preceding section [2.3.3],before the addition of supplementary ingredients.HAT and/or Ouabain were also added in some experiments and this is indicated in the relevant sections.

2.3.5. Fusion of PSA4 and R5/3OA and Isolation of Hybrid Cell Lines

Several methods and modifications of these methods were used before the successful one was developed.These modifications are discussed in Chapter 3 under the heading of "Establishment of a method for Producing Viable Hybrid EC Cell Lines" [section 3.1]. The method which was eventually successful is described below as Method 4.

Method 1:Cell co-cultures were set up in monolayer culture tissue culture bottle in a ratio of 3:1 R5/3OA to PSA4 cells (10^6 cells per bottle) with control flasks (containing R5/3OA or PSA4) also at a density of 10^6 cells per bottle.

Medium was drained off the cells quite throughly and 3-5ml of PEG 6000 was then added,this mixture being kept away from the cells until all of the fusogen

was added.The monolayer was coated completely by gentle rocking of the bottle and the medium aspirated from the cells within 1 minute.This procedure was repeated using successively more dilute concentrations of PEG 6000 (1+3,1+7,1+15) followed finally with two successive washings in complete medium (with or without serum).The control dishes were washed in parallel with successive changes of serumless medium.

Monolayers were then incubated for two hours before they were trypsinised and plated out onto STO feeder cells at a density of $2xI0^5$ cells per 90mm dish and coverslips were also set up from these cells [section 2.3.6].After overnight incubation in complete medium (with 10% FCS) the cells were fed with selective medium containing HAT and Ouabain [section 2.3.1] and the dishes observed for colony survival over the next 1-2 weeks.

Method 2: Dishes were set up as for method 1 but in a ratio of 7:1 R5/3OA to PSA4 cells and were plated out onto pregelatinised dishes ($I0^5$ cells per 90mm dish) after trypsinisation and fed with STO conditioned medium [2.3.3].After 24 hours this medium was exchanged for STO conditioned medium supplemented with HAT and Ouabain to select for hybrid lines.

Method 3: Monolayer co-cultures (and controls) were set up as for method 2 and were treated for 1 minute with a fusogen mixture containing 50% PEG 6000 dissolved in complete medium containing 10% DMSO in the place of serum.Cells were trypsinised after 2-4 hours and plated out onto pregelatinised dishes at a density of 10^5 cells per 90mm dish.Dishes were fed with STO conditioned medium which was supplemented with HAT and Ouabain 24 hours after plating out.

Method 4:Monolayers were set up on gelatin at a density of $2.5x10^5$ cells per bottle for the controls (separate cultures of both parent lines),and at a 2:1 ratio of $6x10^6$ R5/3OA cells and $3x10^6$ PSA4 cells for each of the coculture bottles.The cells were all fed with STO conditioned medium to prevent differentiation of the PSA4 cells.After 24 hours the monolayers were washed twice with Ca^{2+} free medium [Appendix i] and treated with the fusion agent [see section 2.3.2.iii] for 1 minute.After removal of the fusion agent the monolayers were then washed three times with either Ca^{2+} free medium or with PBS.They were then fed with STO conditioned medium and incubated overnight. The cells were then fed with enriched selective medium containing

STO conditioned medium enriched with B-mercaptoethanol,20% FCS,HAT and Oua as described in section 2.3.4.Monolayers were not trypsinised until 7 days after fusion treatment to avoid additional traumatisation of the cells.They were then trypsinised and plated out in the normal way to allow for the isolation of cell lines,using the same selective enriched medium throughout.

When this experiment was repeated several monolayers were set up for parallel fusogen treatment so that a number of independently-arising fusion hybrids could be isolated.

2.3.6. Coverslip Preparations of PEG Treated Cells

Coverslip Preparations of cells from the PEG 6000 experiments were made by seeding out trypsinised cells at a density of 1.6 x 10^4 cells per well into 17mm tissue culture wells into which had been placed plastic thermanox coverslips (approximately 15mm in diameter).These coverslips were either pretreated with Gelatin and the cells plated out in STO conditioned medium,or they were preseeded with an STO feeder layer (8 x 10^4 cells per well).Cells were incubated overnight and the medium was then removed and preparations stained with Leishmans' stain [2.2.8].Coverslips were then removed from the culture wells and allowed to dry.They were mounted,face upwards,onto glass slides and covered with a long (22mm x 40mm) glass coverslip using a few drops of DPX or Coverbond.This gave preparations of better optical quality than if plastic coverslips alone were used.

Slides made from cells treated with PEG 1000 + HEPES buffer [2.3.2.iii] were made by plating out separate co-cultures of ec cells onto the plastic coverslips (1.6 x 10^4 cells per well),these were then treated with the fusogen the following day in parallel with the experimental monolayers []cells were not trypsinised immediately after PEG treatment and therefore it was not possible to make coverslips by simply seeding out a sample of cells from the experiment into coverslip containing culture wells.The coverslips were then stained 24 hours later and mounted as before.

Plastic coverslips prepared in this way were compared with coverslip preparations containing cells from the control,non-PEG treated co-cultures. Coverslip slides were scored by counting the number of Binucleate cells observed,the number of nuclei and the number of cells.Slides were counted

systematically from left to right,choosing several independant microscope fields throughout the whole area of the coverslip.

2.4. Characterisation of Hybrid Cell Lines

2.4.1. Karyotyping

Cells were grown to a late exponential phase in 25 cm^2 culture bottles (approximately 2 days after subculture).At this stage they are fast- growing and therefore producing a large proportion of mitotic cells.The medium was changed 24 hours before they were then incubated in Colchicine (100µg/ml) by adding 0.2mls of the colchicine solution to the 10mls of medium already in the bottle.After 1 hour the monolayer was trypsinised,retaining the medium and PBS washings to stop the trypsin reaction.This avoids loss of mitotic cells which tend to be less well attached to the growing surface than cells in the rest of the growth cycle.

Cells were then spun down (1000 g,3-5 mins) and resuspended in hypotonic KCl (0.075 M) to swell the cells.After a 4 minute incubation at room temperature the cells were again spun down and all but a drop of the supernatant was aspirated off the cell pellet.The cells were then resuspended in this drop of liquid by flicking the tube which was then placed on ice.

The cells were fixed in freshly made,ice-cold,Methanol:Glacial Acetic Acid (ratio 3:1),adding dropwise with a pasteur pipette 10ml of the fixative to the cells mixing by flicking the tube after each drop is added.The preparations were then left on ice for 30 minutes after which time they were again spun down (1000rpm,3-5mins) and fixed in a further 10mls of fixative for 5 minutes on ice.After spinning down and removing this final fixative the cell pellet was then resuspended in about 0.5ml of fixative (depending on cell density) and slide preparations were made by dropping the cell suspension onto washed slides held at an angle of 45 degrees and with the pipettes end about 12 inches from the slide.This ensured that the cells in metaphase were well spread out,allowing the separation of chromosomes.

Slides were cleaned by standing them overnight in Chromic acid followed by all day washing in running tap water.They were then stored in 70% alcohol until

required.Prior to being used for karyotyping they were rinsed for 30 minutes in tap water and allowed to dry.

Once made slides were either immediately stained 2 minutes in Giemsa (7.5 g/l in Glycerol/methanol 1:1 v/v) or in Leishmans stain (a stock solution [1.5 g/l in methanol] was diluted with 3 volumes of Gurrs buffer pH 6.8 prior to use),most being stained in Leishmans as this showed up the chromosomes more clearly than did Giemsa staining .Alternatively some of the slides were left for 7-14 days to dry out completely and were then either trypsin or centromere banded [see next section,2.4.2].

2.4.2. Banding Chromosomes

i)Trypsin Banding Slides of metaphase spread chromosomes prepared as above [section 2.4.1] were flooded with trypsin solution (0.05% w/v trypsin,0.02% w/v EDTA,in PBS) and incubated at room temperature for 1-3.5 minutes depending on the ambient temperature and the slides.To control this a test slide was done each time the banding was attempted and the buffer and trypsin solutions were allowed to equilibriate to room temperature before use.After incubation in the trypsin the slide was washed with Gurrs buffer solution (pH 7.2) and the slide was then flooded with pH 6.8 Gurrs buffer.Finally the slide was stained for 3 minutes with Leishmans stain (1.5g/l in methanol,used as a 25% solution in Gurrs buffer pH 6.8).

ii)Centromere Banding Slides,preferably 2-3 weeks old,were placed in 0.2N HCl for 1 hour at room temperature.After rinsing in DDW (deionised distilled water) the slides were then treated with 5% $Ba(OH)_2$ either for 5-15 minutes at room temperature or for 10-15 seconds at 50° C,whichever was the most convenient.(If this was done at the higher temperature then extreme caution was required as $Ba(OH)_2$ is highly corrosive.)Finally the slides were rinsed several times in DDW and were then left for 1 hour in 2xSSC (0.3M NaCl,and 0.03M Na_3 Citrate).The slides were then stained with 10% Giemsa for 30 minutes.

2.4.3. Toxicity Tests

To determine the resistance or susceptibility of cell lines to a soluble chemical,cells were usually plated out at cloning density (10^3 cells per dish) in 60mm tissue culture dishes and then fed with medium supplemented with the chemical under test for a specified number of days.The dishes were then stained with Leishmans stain [normal stock solution,section 2.2.8] and the plating efficiency estimated by counting of colonies.

This basic method was used routinely for testing the ability of cell lines to grow in either HAT,Ouabain,or both of these using the same concentrations as described for selective medium [section 2.3.1] and using as a control,dishes of the same cell line set up in parallel with the test dishes and fed with the normal culture medium.

Thioguanine toxicity was tested in a similar way using several different concentrations of thioguanine to ascertain an LD_{50} value.LD_{50} was defined as the concentration of test chemical required to reduce cell survival to half that of the control,it was estimated by plotting the colony count results of the toxicity experiment against concentration of 6-Tg used and then finding the point on the graph which coincided with a 50% reduction in colony counts.

Dishes of test cells were set up and fed with medium containing concentrations of 6-thioguanine which increased from 0 µg/ml to 100 µg/ml over 6-7 different test dishes.These were then incubated for 5-10 days,feeding with 6-Tg supplemented medium as required,then stained and counted as usual.

2.4.4. Analysis of Metabolic Cooperation - ^{3}H-Uridine Transfer

Cells were seeded onto pregelatinised 30mm tissue culture dishes at a density calculated to give sufficient cells for contact,but not too many to cause overcrowding [126,modified].The number of cells plated therefore depended on cell size:for small cells such as Modl and PSA4 plating density was $2x10^5$ cells per dish,while larger cells such as R5/3OA and the Hybrid lines were plated at a density of $5x10^4$ cells per dish.As well as the test dishes (set up either in duplicate or in quadruplicate) a recipient control dish was set up for each line

tested.The donor cell dishes were set up at the same density as the recipients.

After incubation overnight,the donor cells were fed with medium (0.5ml per dish) supplemented with 10μCi/ml of ^{3}H-Uridine for a period of 3 hours.The isotope was then washed off the cells by three changes of PBS and the cells were trypsinised.Donor cells were seeded onto the recipient dishes at a density of $1/5^{th}$ of a dish of donor cells per dish of recipients.A donor cell control was set up in a freshly gelatinised dish at the same density.The experiment was incubated (37° C,5%CO_2) for 3 or 4 hours as stated in the results section.

The medium was then removed from the dishes and they were each given a number,the dish number being recorded against the corresponding cell line(s) in a key.They were then washed twice with PBS and fixed by inversion over tissue soaked in freshly made Methanol:Glacial Acetic Acid fixative (ratio 3:1) enclosed in an air-tight container.The dishes were normally fixed overnight,after which time free nucleotides were removed by treating the dishes with ice-cold 10% TCA (in DDW),this TCA treatment was done twice,10 minutes each time,at 4° C.

Dishes were then washed for approximately 12 hours (either all day,or overnight) and then allowed to dry completely in air at room temperature for at least 24 hours.The photographic emulsion could then be applied,in the dark room using only the illumination of the red safety light.Photographic emulsion was melted in the 50° C water bath and diluted with water in a ratio of 1 part emulsion to 1.5 parts water by volume.The cell surface of the dishes was then coated with a thin layer of emulsion by pouring about 2ml of this solution into the dish and then draining off thoroughly.When all the dishes had been coated in this way they were dried for at least 1 hour in a stream of cool air from an electric hairdryer.Once dried they were placed in air and light tight tins or boxes together with small packets of silica gel ,wrapped up in foil and sealed,and then stored in the cold room at 4° C until ready for developing.

The time taken for grains to develop varied considerably depending on the batch of ^{3}H-Uridine used,variations of serum and other culture conditions.Therefore two control dishes were set up,containing donor and recipient cells,and these were developed 7-10 days after the emulsion was put on to determine whether or not to develop the experiment.

Dishes were developed for 3 minutes in D19 developer(Kodak) made up as

instructed on the packet.They were then rinsed in water and fixed for 20 minutes in Amfix fixative,diluted 1+3 with DDW.After this time they were no longer light sensitive and were washed for 20-30 minutes under gently running tap water.Finally dishes were lightly stained with leishmans (2 minutes instead of 5,see section [2.2.8]) and could then be scored.

When the stained dishes had dried the sides were removed to facilitate microscope observation and coverslips were applied with a drop of immersion oil,as the slide mountants available all either corrode the plastic dish or cause diffusion of the stain.The cells were then observed under the light microscope using the x100 oil immersion lens.

Dishes were scored by counting 100 pairs of cells per dish.One cell of each pair being a recipient cell in contact with a donor cell (easily distinguishable by the heavy ^{3}H-Uridine labelling),while the other cell of the pair was a control recipient cell which was not in contact with a donor cell.This system controls for variability in background count across the dish.

Dishes were counted using the number code only and this was compared to the key when the experiment has been counted.This method avoids some of the bias which can occur when the cell line being scored is known.

Results were presented in Histogram form and were also analysed by means of a computer programme designed by T.Smith [155].This estimates the percentage of contacts manifesting communication by calculating a frequency distribution of the differences in the counts between all possible pairs of one contacting and one isolated recipient (10 000 pairs in all per dish) and this calculation was then transformed by subtracting the frequencies for negative differences from those observed for positive differences of equal magnitude.The fraction of cell-cell contacts showing cooperation being estimated as the ratio of the area beneath the transformed frequency distribution to that beneath the untransformed curve.

An alternative method for the estimation of the extent of cooperation was also used in this study.This measurement (T-NT/X) was calculated from the subtraction of the median of grain counts for cells isolated from donor cells (NT) from the median of the grain counts from contacting cells (T).The median of both T and NT was calculated by the computor programme. The value T-NT was then adjusted for ploidy (X) because ploidy affects cell size and cell size is

directly related to the grain count value per cell which is used to estimate the extent of cooperation.It is important to adjust for ploidy when comparing the cooperation ability of cells with greatly different ploidy such as that of the diploid line PSA4 and that of the sub-hexaploid line PR3.

2.4.5. Analysis of Embryoid Bodies

Embryoid bodies were made according to the protocol outlined in section 2.2.10. and were analysed for the extent of differentiation using the following classification system.This places each embryoid body into one of five classes which were determined by the amount of differentiation observed according to the extent of parietal and visceral endoderm development,eosin (red) staining,and formation of cavities.

The Figure 2:1 illustrates the various stages of embryoid body differentiation identified.Slides of sectioned EB's were scored without knowledge of the cell line from which they were derived (see scoring of Uridine transfer analysis experiments [2.4.4]) and each EB was placed into one of the 5 categories.Results were then expressed as percentages of EB's presenting each category and illustrated in a pie chart.The greater the proportion of EBs in the later categories (iii-v),the greater the differentiatative potential of the cell line.

2.4.6. Tumour Formation

The ability of cell lines to form tumours was assayed by the injection of live cells ($2x10^6$ cells in 0.3mls CM + 1% serum,per mouse) into syngenic mice.Therefore the Hybrid cell lines which were derived from PSA4 and R5/3OA,both of 129 origin,were injected into male and female (129) mice,whereas the Modl cell line was derived from C57Bl stock and therefore was injected into mice of the C57Bl.Modl^{null} mouse strain.

The cell suspension was injected subcutaneously into the back of the mouse's neck (0.3ml per mouse) and the animals were thereafter observed for the development of tumours.Usually within 2-4 weeks of injection tumours began to form,but some tumours took much longer to form,therefore the mice were monitored regularly for the next 9-12 months after which time they were killed and autotopsied for the presence of tumours and other possible abnormalities.

When a tumour was detected in a mouse the animal was observed closely and was killed when the tumour size was approximately 1.5 cm across.The tumour was dissected out and sliced into five.Three separate slices were then taken and fixed in Bouins solution for paraffin sectioning.The remainder of the tumour

Figure 2:1

CLASSIFICATION OF EMBRYOID BODY DIFFERENTIATION

Illustrates the four differentiated categories (2–5) and the two undifferentiated categories (1.1 and 1.11).

1.1 Completely undifferentiated embryoid body stained predominantly with haemotoxylin but with an outer ring of eosin staining,necrotic looking cells which could not be positively identified as endoderm differentiation.This category was found only in embryoid bodies of poorly differentiating lines.

2. Predominantly staining purple (haemotoxylin) but with a well defined endoderm ring (eosin staining) and small patches of eosin staining in the centre of the body.Both PE and VE identifiable.

3. As for **2.** but with far more extensive internal differentiation and less haemotoxylin staining.

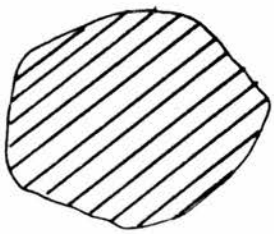
4. Well differentiated endoderm and the beginnings of cavity formation generally containing eosin stained necrotic looking cells.

5. Large,fully formed cavity with well developed parietal and visceral endoderm and extensive eosin staining of differentiated areas.

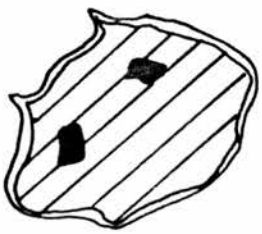
Haemotoxylin staining -

Eosin Staining -

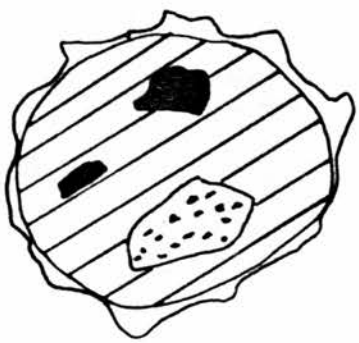
1(1) Undifferentiated



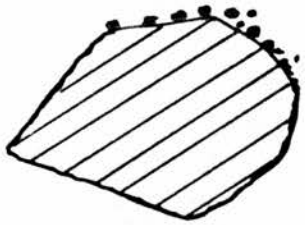
2 VE and PE +



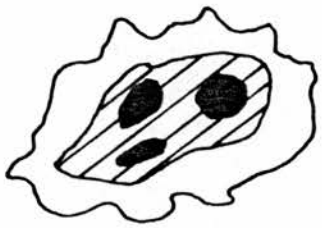
4 VE,PE and Cavity +



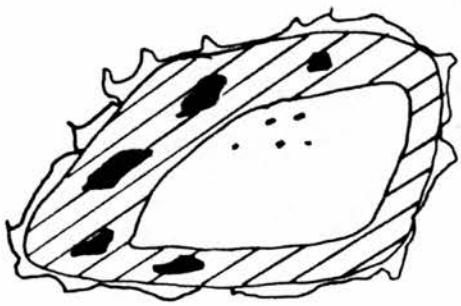
1(11) Undifferentiated



3 VE and PE ++



4 VE,PE and Cavity ++



was either discarded or used as a primary explant for the isolation of cell lines [see section 2.5.5].

2.5. Isolation of Derivatives of Hybrid Cell Lines

2.5.1. Thioguanine Resistance

90mm dishes of the Hybrid PR3 were plated out at densities of both 3000 cells/ml [63] and also at the lower density of 100 cells per ml. Dishes were then fed with either 10µg/ml, 20µg/ml or 30µg/ml of 6-Tg,for approximately 10 days after which time the suviving colonies were incubated in CM10 until they attained sufficient numbers to be picked off and transferred to cloning wells from where they were grown up into permanent stocks.

2.5.2. "Kiss of Death" - Selection of Non-Cooperating Lines from Thioguanine Resistant Hybrid Variants

Pre-gelatinised dishes (90mm) were set up containing cocultures of PR3 and PR3Tg12 cells in a ratio of 1:1 and at a cell density of 7.6 x 10^5 cells per dish [60].They were fed with STO conditioned medium [section 2.3.3] and incubated overnight.The medium was then exchanged for conditioned medium supplemented with 10µg/ml 6-Tg [section 2.3.1] and the dishes incubated for approximately 10 days until the majority of cells had been killed and a number of surviving colonies were to be seen.These colonies were then grown up in STO conditioned medium until they were large enough to be transferred to cloning wells and grown up into permanant stocks.

This procedure was repeated once more,using a line selected in the first round of Kiss of Death selection rather than PR3Tg12,to further enrich the population with non-cooperating cells.

2.5.3. Isolation of Cell Lines with Reduced Differentiation Ability using STO Conditioned Medium

The development of this assay is described in detail in the results section [5.1.].Cells derived from the Hybrid lines were set up at cloning density (10^3

cells per dish) in STO conditioned medium [2.3.3] on pre-gelatinised 90mm dishes.Growing colonies were then observed and those colonies which did not appear to be exhibiting any differentiation,by phase microscope criteria,were picked off when large enough and plated out into cloning wells from where they were grown up into permanent frozen stock.

2.5.4. Establishment of Cell Lines from Mouse Experimental Tumours

Cell lines were isolated from slices taken from Hybrid cell line induced teratocarcinomas.The sections were placed aseptically directly into a sterile plastic tube,from where they were transferred onto tissue culture dishes containing STO feeder layers as quickly as possible,the tissue being broken up with the aid of a sterile pipette and a small quantity of PBS.The dish was then observed over the next few days and if there were signs of ec cell-like growth then these cells were grown to confluence and a frozen stock made.

2.6. Isolation of Cell Lines from Mouse Blastocysts

2.6.1. PSA4 Conditioned Medium Dialysis,and preparation of Dialysis Tubing

Dialysis tubing was cut to length and then was boiled in 0.5M $NaHCo_3$ for 30 minutes.The tubing was then washed twice in distilled water and then boiled for an additional 30 minutes in 0.5M K_2EDTA.This was followed by a thorough washing in distilled water,at least 3 changes,and the tubing was then stored completely covered in water at room temperature, until required.

PSA4 conditioned medium [99] was prepared from the supernatant taken from monolayer cultures of growing PSA4 cells at a cell density of 10^7 cells per 90mm dish.STO feeder contamination was reduced by using cultures which had been previously enriched for PSA4 cells.This was done by allowing a suspension of trypsinised PSA4 cells (plus residual STO feeder cells) to settle for 30 minutes(37° C,5%CO_2) after which time very few PSA4 cells but the majority of STO cells will have attached thus leaving most STO feeder cells behind when this PSA4 enriched cell suspension was replated out onto gelatinised 90mm dishes.

The PSA4 cell monolayers were then incubated for a further 2 days,after which

time the culture medium was exchanged for CMX [appendix i] and the cells incubated for a further 48 hours.This medium was then removed from the cells and spun down (1000 rpm,3-5minutes) to remove cell debris.The supernatant was then further purified by dialysis (5 litres of 20mM NH_4HCO_3 per 200ml of medium,48 hours,at 4° C).Dialysis buffer was changed at intervals of 4- 6 hours throughout the dialysis period.

The dialysed medium was lyophilised and the resulting powder was then reconstituted in 10 ml of CMX (per 200 ml of medium dialysed) and sterilised using a 0.2υm millipore filter.This concentrate,supplemented with 10% FCS and 10^{-4}M B-mercaptoethanol was then stored at -70° C until required for use.When added to medium it was regarded as an approximately 20 fold concentrate and was therefore used in medium as a 5% supplement.

2.6.2. Superovulation,Mating and Plugs

Female mice were superovulated by injection of PMSG (5 i.u. per mouse,in 0.5ml PBS) between the hours of 12 noon and 4pm followed by a similar injection of HCG (5 i.u. per mouse,in 0.5 ml PBS) 48 hours later.[47,and personal comm.from R.G.Gosden]

Mice were mated with suitable males after the second injection,and examined for copulation plugs the following morning.Those mice successfully mated were separated and the females kept until the embryos were of the age required.The mating plug was detected with the aid of a mounted wire which could be inserted into the cervix of the animal.If the passage of the wire was impeded then a plug was present and the mouse was taken to be approximately 12 hours pregnant.

2.6.3. Removal and Culture of Mouse Blastocyst Embryos

Early blastocyst embryos were flushed from the uterus of mated female mice,73-79 hours after the detection of the mating plug.Day 3.5 mice were killed by cervical dislocation and the uterus dissected out.The embryos were removed as illustrated in Figure 2:2.Embryos were manipulated with a mouth controlled pipette which had been sterilely drawn out just prior to use,to the required internal diameter (for manipulation of blastocysts this was 110-130

µc and for flushing out uteri or oviducts the micropipette was made slightly shorter and therefore wider).This was done in a laminar flow hood using a bunsen burner .

Paraffin oil was sterile filtered with an oil resistant millipore filter, after first prefiltering with an 0.8 υm millipore filter,and equilibrated with 3-5ml of medium and 5% CO_2 in air for 2-3 days prior to use. Using the Binocular microscope for magnification,embryos were removed separately for incubation into small droplets of CM10 (FCS) placed under equilibrated paraffin oil.They were then usually cultured to the late expanded blastocyst stage in vitro.

Mouse embryos up to the morula stage require specialised media for their in vitro culture but it was possible to grow embryos of the late morula to late blastocyst stage in normal tissue culture medium supplemented with FCS and B-mercaptoethanol.The antibiotics Penecillin (5000 iu/ml) and Streptomycin (5000 mcg/ml) were also added in early experiments to reduce bacterial contamination although with improved experimental technique they were found to be unnecessary.

2.6.4. Removal of the Zona Pellucida

Two methods have been used for removal of the Zona Pellucida of blastocyst embryos,the Pronase method being the easier and more effective,and therefore this latter method was adopted for all later experiments.

i) Acid Tyrodes solution [55] is an acid salt solution containing,per 100ml distilled water,NaCl (0.8g),KCl (0.02g),$CaCl_2$ (0.02g),$MgCl_2.6H_2O$ (0.01g),$NaH_2PO_4.5H_2O$ (0.005g),Glucose (0.1g),$NaHCo_3$ (0.1g),PVP (0.25g).When the salts have dissolved the pH of the solution is acidified to 2.5 using 5N HCl.It was then sterilised by autoclaving,in a closed bottle to prevent altering of the PH.

To remove the ZP embryos were placed in a small drop of the Acid Tyrodes solution and incubated for 5-15 minutes [146].

ii) Pronase [72,117] method of ZP removal involved a 15 second incubation (37° C/5% CO_2) of the embryos in 0.5% pronase dissolved in Tris-Citrate buffer (pH 7.0).

2.6.5. Isolation of ICM from Blastocysts using Immunosurgery

Early blastocyst mouse embryos were flushed from the uterus of pregnant superovulated females [2.6.3] and cultured overnight under paraffin to the fully expanded blastocyst stage [99].The ZP was then removed using pronase [55] (or Acid Tyrodes solution) as described in the previous section [2.6.4].Embryos were then preincubated for 15 minutes in an antibody to mouse cells [2.6.7],at 37° C and 5% CO_2,followed by three 5 minute washings in PBI + 10% FCS.Embryos were then transferred to the complement solution [2.6.6] and incubated 45 minutes at 37° C and 5% CO_2 [158].

All incubations and washings were carried out sterilely,in small droplets placed in a plastic petri dish,using a mouth pipette with a drawn pasteur and the binocular microscope as described [section 2.6.3].After the incubation in complement the intact ICM was gently teased from the lysed trophoblast cells and,using a slightly narrower gauge drawn pasteur pipette [2.6.3] than for whole embryo manipulations,was then transferred into cloning wells for further culture.

2.6.6. Preparation of Complement

Human complement was prepared from whole blood as follows:420 ml of blood was taken into a serum pack (by staff of the Blood Transfusion Unit,Edinburgh) and hung overnight in the cold room (4° C) so that the serum is separated from the cells by being allowed to drip into the attached second pack.The serum was then spun down (1000 g for 15 mins) and the supernatant transferred as quickly as possible into Beckmann tubes in 1ml quantities.The Complement began to degrade after 24 hours at room temperature and so it was necessary to transfer the tubes of serum into the liquid nitrogen freezer as soon as they had been aliquoted.

Complement used in immunosurgery was initially from my own blood but when this was shown to cause problems [7.1] complement from Dr Stewart Blackie of the Pathology Department was used instead.Later it was found that Guinea Pig Serum obtained from the Scottish Antibody Production Unit (SAPU) also

Figure 2:2

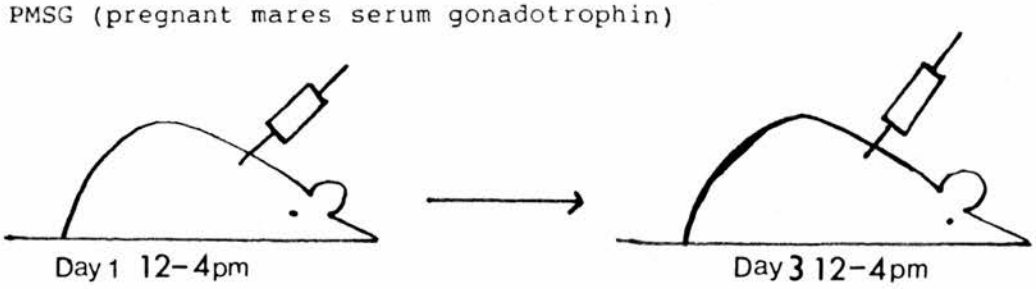
SUPEROVULATION,REMOVAL OF MORULA EMBRYOS AND ISOLATION OF CELL LINES FRM MOUSE BLASTOCYSTS

Illustrates the sequence of events from mouse superovulation to removal of morula embryos from the uterus and their subsequent culture to the blastocyst stage.

Blastocyst embryos are then used in tissue culture to obtain cell lines as shown.

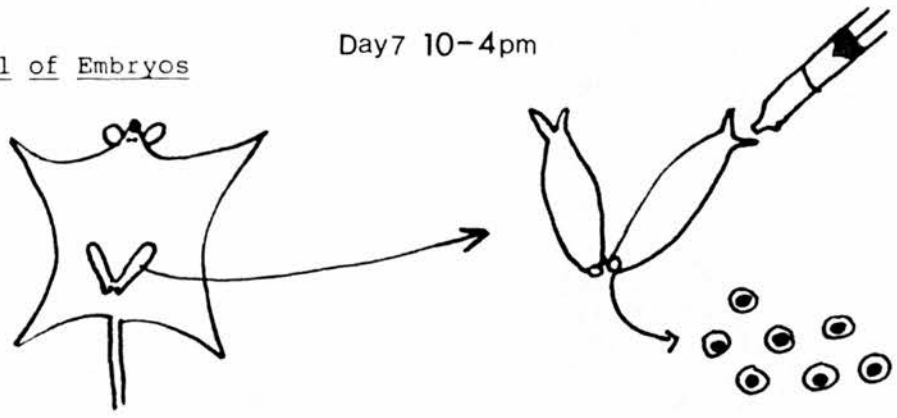
Superovulation

Female mouse is injected with 5i.u. of PMSG (pregnant mares serum gonadotrophin)

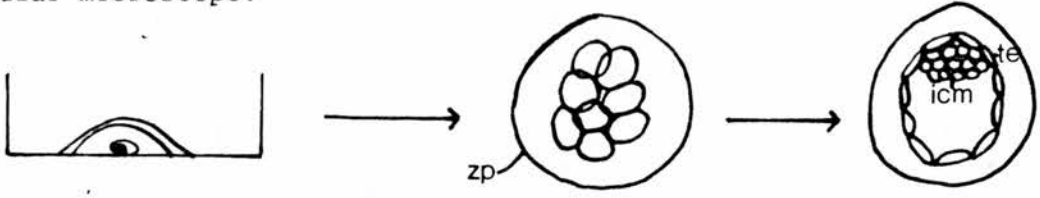


The same female mouse is injected with 5i.u. of HCG(human chorionic gonadotrophin),and is then caged with a single male C57 Black Mod1^{null} mouse.

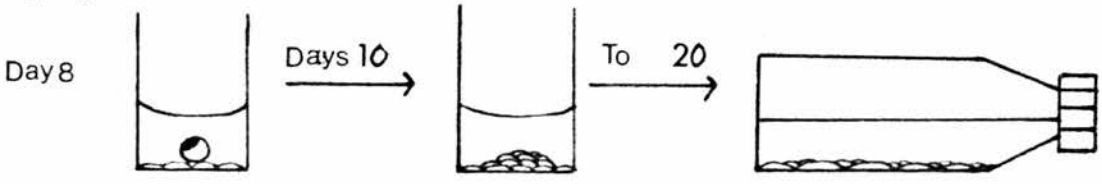
Removal of Embryos



All femal mice which showed mating plugs on Day 4 are killed by cervical dislocation,and the uterus removed intact.Morula and blastocyst embryos are then flushed out of the uterine horns with the aid of a mouth pipette and binocular microscope.



Embryos are then cultured overnight (37°C,5%Co_2) in microdrops of 20% fetal calf serum supplemented eagles tissue culture medium,with B-mercaptoethanol,under paraffin oil equilibriated to 5%CO_2 to prevent drying out.



When fully expanded blastocysts are formed,the zona pellucida was removed by a 5-10minute treatment with 0.5% pronase in Tris-citrate buffer (pH 7.0) and embryos plated out,intact,into 'Optikon' wells containing a feeder layer of mitomycin-C killed STO cells.Outgrowths were grown up and a stock eventually frozen down.

provided an adequate source of complement and this was used in all subsequent experiments.

2.6.7. Agglutination and Lysis Test for Antibodies and Complement Used in Immunosurgery

Mouse blood was obtained from a stock mouse either by bleeding from the eye or the tail and was used as the source of mouse cells for the purpose of testing antibodies and complement.Blood was taken into a tube containing Alsever's solution to prevent clotting and was then spun down (1000 g for 10 minutes),washed three times in PBS and then resuspended in concentrated form to give a 2-4% suspension of red blood cells.

Using a plastic tray of wells,which were covered during incubation periods to prevent condensation interfering with the reaction,doubling dilutions were made of all the test antibody sera both with and without a standard volume of complement (0.1ml).Doubling dilutions were also made of the complement sera to which were added a standard volume of antibody serum (0.1ml) and a control series of wells which contained the saline (PBS) only.

When all the wells had been set up the reaction was started by adding 0.1ml of the freshly prepared concentrated suspension of mouse red blood cells and the tray of wells was incubated for 45 minutes at 37° C.The wells were scored as illustrated in Table 2:3 and the results analysed for the presence of antibody and/or complement activity.

2.6.8. Establishment of Cell lines from Embryo Outgrowths

Several different methods and modifications to published methods were necessary to determine the following method for establishing cell lines from blastocyst embryo cells and these are described in the results [7.1].

Either ICMs,isolated using immunosurgery [2.6.5],or whole embryos,with or without their ZP removed,were plated out individually onto STO feeder cells (5000 cells per well) into Optikon cloning wells (one embryo or ICM per well) and were then fed with very small quantities (20ul or less) of enriched medium [2.3.4] so that the growing cells had the chance to condition the medium in

which they were growing.

When the embryo (or ICM) had produced a large clump of outgrowth cells and appeared to be growing well the outgrowth was trypsinised and the cells transferred to a larger cloning well,preseeded with feeder cells.When these wells had become confluent a permanent cell line was made by transferring the cells to 25 cm^2 culture flasks containing feeder layers and frm there they were grown up and frozen down as usual [2.2.7].

Medium changes were made by exchanging only 50% of the medium at any one time while the cells were still in small quantities.This allowed a reasonable compromise between replacing depleted essential nutrients from the old medium and retaining enough of the growth aiding conditioning agent(s) built up in this medium by the growing cells.

2.7.Characterisaton of Mod1

2.7.1.Histochemical Stains

a) Alkaline Phosphatase: A cell monolayer (or slide of sectioned cells) was washed with PBSA and then fixed for 30 minutesat room temperature in Formol Saline.Meanwhile the staining solutions were prepared as follows; Solution A - 4g New Fuchsin was dissolved in 100ml of 2M HCL at 60°C,it was then filtered before use (this solution was stable for several weeks).Solution B - 4g $NaNO_2$ was dissolved in 100 ml H_2 this was stable at room temperature for only 48 hours).

To use the stain,equal volumes of solutions A and B were mixed,0.2ml of the resulting mixture was then added to 40ml of 0.2M Trs-Cl at pH 9.2.To this was added 5mg of Napthol AS-MX Phosphate in 0.2ml dimethyl Formamide.The whole mixture was then filtered and used immediatedly.

The stain mixture produces a red colouring of cells containing alkaline phosphatase,after allowing 30 minutes (37°C) for thereaction to work.

b) Malic Enzyme: (Prof.Gardener) Stock solutions of the following were made up in advance and stored in the fridge : Nitroblue Tetrazolium (NT) 1mg ml in distilled water,0.2M Tris-HCl buffer at pH 7.6,Manganous Chloride 0.9mg ml in distilled water,Meldola blue 0.5mg ml in distilled water and Phenazine

Methosulphate(PMS) 30.6mg/ml in distilled water (the PMS was light sensitive and therefore was stored wrapped in tin foil).

A stock solution of 1M L-Malate was made up by dissolving 1.561g of solid L-Malate in 8ml distilled water.40% NaOH was then added dropwise until the solution was just on the alkaline side of PH 7.The solution was then made up to 10ml with distilled water and frozen as 0.1ml aliquots.

The following quantities of stock solutions (2.5ml NBT,2.5ml Tris HCl,1.0ml $MnCl_2$,3.0ml distilled water,1.0ml Na Azide,and a 100µl aliquot of L-Malate) were mixed with lmg of NADP per ml of final staining solution,and after thorough mixing the pH was adjusted to 7.6 with either 0.2M Tris or 0.2M HCl.Finally,just prior to use 0.5ul of PMS per ml of staining solution was added,unless Parietal Endoderm was to be stained when the PMS was replaced with 0.5µl of Meldola Blue per ml of staining solution.

The cells to be stained were grown in monolayer culture on sterilised glass coverslips.When confluent the medium was removed,the cells washed in two washes of PBS and the preparations fixed for 2 minutes in 0.6% crude Glutaraldehyde (BDH 25% aqueous).If the fixation was unsatisfactory it was possible to vary the Glutaraldehyde concentration between 0.1% and 0.6% and the fixation time between 1-4 minutes.The fixed coverslips were rinsed in PBSA for 36-48 hours at 4° C and were then placed in a plastic dish and stained with the staining mixture prepared as above,using 3-5mls of stain per coverslip.

The stain is left on the cells (at 37° C/5% CO_2) until differential staining was visible,this process can take up to 36 hours.If malic dehydrogenase was present a grainy purple staining appeared in the cells cytoplasm and nucleus,some staining will also appear in the Null cells but to a much lesser extent.

The preparations were then fixed in Carnoys fixative (ethanol 60%,chloroform 30%, and Glacial Acetic Acid 10%) after first removing them from the plastic dishes which dissolve in Carnoys,and were finally then mounted on glass microscope slides using DPX.They were then viewed under the light microscope.

Table 2:3

SCORING OF AGGLUTINATION AND LYSIS TEST FOR SERUM SOURCES OF ANTIBODY AND COMPLEMEN

Shows the criteria employed in categorising the results of incubating antibody and or complement with a mouse red blood cell (rbc) concentrate.

Result	Appearance of Well	Conclusion
No Reaction	rbc's have normally sedimented to the bottom of the well	Antibody not present or too dilute for a reacti
Agglutination	No red blood cell sedi-ment and clear supernatant which may be colou-	Antibody present at the correct dilution
Lysis	Clear and colourless supernatant and tightly packed"spot" at the bottom of the well	Complement and antibody present at the correct dilutions

2.7.2. Enzyme Assays

a) Alkaline Phosphatase Assay

Cell pellets were prepared by trypsinising nearly confluent medium sized (75 cm^2) culture bottles of cells,stopping the reaction with CM.Cells were then washed twice in CM by centrifugation (1000 g,3 minutes) followed by two washes with 0.9% (isotonic) NaCl.Cell pellets thus prepared were then either used immediately in the enzyme assay or were stored at -20° C in their plastic conical flasks.

When required for assay pellets were thawed and diluted with 0.2ml distilled water.Samples were then sonicated for three 10 second bursts,cooling with iced water in between bursts.The mixture was then centrifuged at 3000g for 15 minutes .The supernatant constitutes the extract and is then assayed as described below.

A reaction mixture (0.5ml) was prepared which contained the following,p-Nitrophenyl Phosphate (10mM),$MgCl_2$ (2mM),2-amino-2,methyl propanol/HCl buffer (0.5M,pH 10) and an appropriate amount of extract,determined by protein assay and described below.After 30 minutes incubation at 37° C the reaction was stopped with 1.5mls of 0.25 NaOH and the mixture was then diluted to 3.5mls with distilled water.The results were read on a spectrometer at an optical density of 410nm (OD410) against a blank made up of the reaction mixture containing an equivalent amount of distilled water instead of the cell extract.

b) Protein Assays

The method normally used for assaying protein was the Biorad method [16],but occasionally this method was not available and the Lowry method was used instead [91].

i) Biorad Assay Protein standards were made up by dissolving 1mg Bovine Serum Albumin (BSA) into 1ml of distilled water,dilutions were then prepared (total volume 100µl) containing from 0-100υl of the BSA solution.Test samples were made up to be in the same range as the standard,therefore if a cell lysate was used samples containing 2υl,5υl and 10υl of extract made up to 100υl were used.An example of a Biorad protein standard curve is illustrated in

Figure 2:3

To each test-tube containing protein standard,test or control was added 5ml of the dye reagent Coomassie Brilliant Blue G-250 (100mg) dissolved in 50ml 95% ethanol.To this was added 100ml 85% w/v phosphoric acid,the resulting solution then being diluted to 1 litre using DDW.Final concentrations in the reagent were therefore,0.01 w/v Coomassie Blue,4.7% w/v ethanol and 8.5% w/v Phosphoric acid).This solution is now available commercially as a concentrate which is diluted 1+4 before use.The tubes are then covered with film and mixed by inverting (vortexing was avoided as it caused foaming of the BSA solutions).After 15-20 minutes at room temperature the optical density was measured at 595nm against the control tube which contained the reaction mixture and DDW only.

ii) Lowry Assay reagents were made up as follows;Solution A 50ml,2% Na_2CO_3 in 0.1N NaOH;Solution B 1ml,1% $CuSO_4$ + 1ml,2% Na_2 Tartrate;Solution C 50ml Solution A + 1ml Solution B;Folin Reagent,equal parts of Folin concentrate (BDH) and distilled water;Albumin Standard 1mg/ml in distilled water. Reaction tubes were set up as shown in Table 2:4 and the optical densities of the final reaction mixtures were then read at 750nm against the blank control.

c) Assays for Plasminogen Activator

i) Fibrin Gel Overlay assay.Cells were plated out in duplicate onto 60mm dishes and allowed to form colonies.When slow growing cell lines such as Modl were to be compared with fast growing lines such as PYS this entailed staggering the plating out over several days to achieve similar densities of cells in the different dishes.

Then,using a modification of the Fibrin Gel method of Strickland and Mahdavi (ref) cultures were washed twice with PBS and then overlaid with 1.5ml of the reaction medium made up as follows;PBSA (1.1ml),Plasminogen (3.5ml,containing 6 units),Fibrinogen (5ml of a 1% solution made up in distilled water),10x GMEM (1ml),Bicarbonate (0.37ml),Glutamine (0.11ml),and DDW (3.9ml).Thrombin (20 units/ml) was added just before use.In control dishes the plasminogen was replaced by PBSA.The plasminogen was freed from low molecular weight material prior to addition to the reaction medium by gel-filtration on a Sephadex G25M column.

Figure 2:3

STANDARD PROTEIN CURVE – BIORAD ASSAY

Samples of test cell lysates were read against the curve produced by test readings containing from 1–100µg of BSA (bovine serum albumin) protein.

The standard was made separately for each new experiment as results were shown to vary.

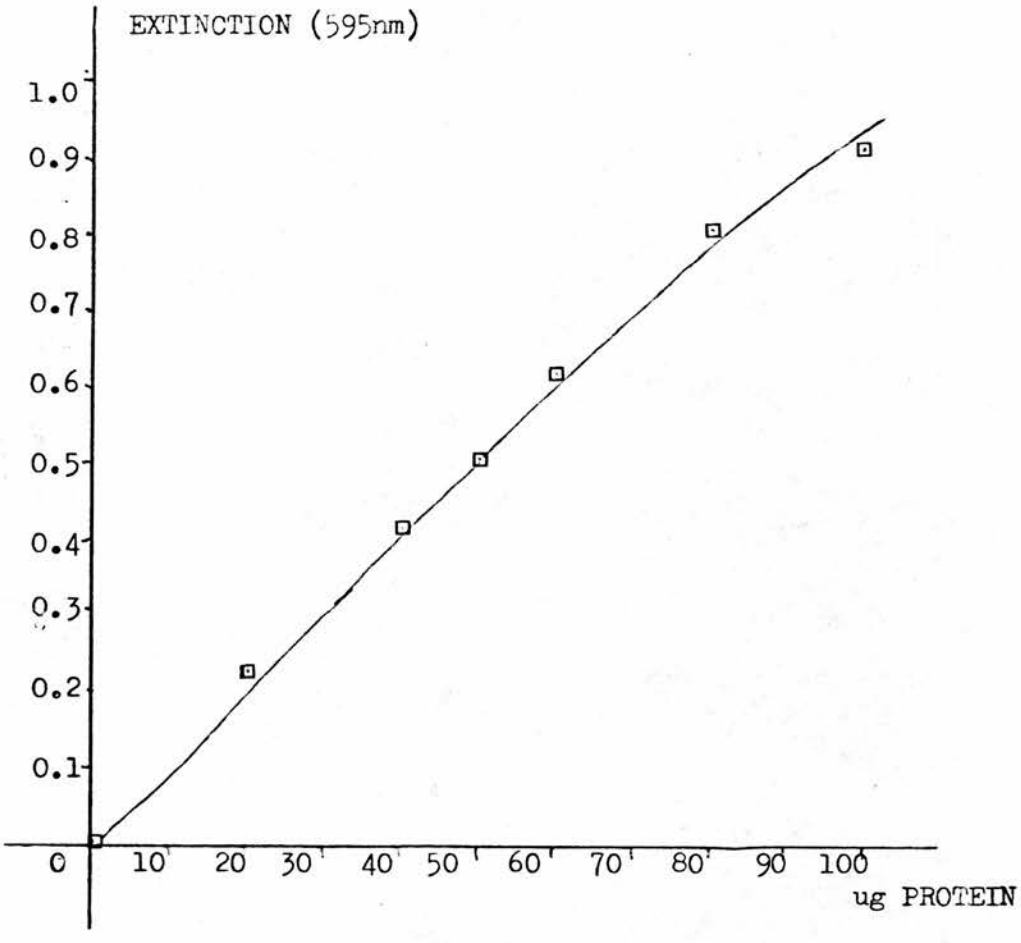

EXTINCTION (595nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
90
100
ug PROTEIN

Table 2:4

LOWRY METHOD FOR PROTEIN ASSAY

Shows the protocol and reagents used in the Lowry method of protein assay which was used as an alternative to the biorad assay when this latter was unavailable.

Reagent	Standards				Tests		Blanks	
	1	2	3	4	1	2	1	2
Albumin(ml)	0.2	0.1	0.05	0.025	-	-	-	-
Test Cytosol(ml)	-	-	-	-	0.2	0.1	-	-
Distilled Water(ml)	0.2	0.3	0.35	0.375	0.2	0.3	0.4	0.4
Solution C(ml)	3	3	3	3	3	3	3	3
Vortex and leave for 10 minutes								
Folin	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Vortex immediately and leave for 30 minutes								
Final reaction mixture is then read in the spectrophotometer at 750nm								

ref:Lowry,O.H.,Rosebrough,N.J.,Farr,A.L. and Randall,R.J.(1951). J.Biol.Chem.**193** 265-275.

When the dishes have been overlaid a fibrin gel was formed almost immediately.The dishes were then incubated at 37° C/5%CO_2 for up to 4 hours,although they were checked at regular intervals for lysis zones as cell lines such as Modl and PYS were found rarely to require more than 1-2 hours for well defined lysis to have occured.After incubation the gels were fixed in methanol and then stained with Kenacid Blue (2mg/ml in Methanol/Glacial Acetic Acid/Water,45:10:45) to enable clearer visualisation of the lysis zones.

ii) Fibrin Layer Assay,fibrin gels (prepared by Yvonne Barlow,Dept.of Oral Medicine and Oral Pathology,Edinburgh) were set up in 60mm dishes.Samples of conditioned medium prepared by incubating cell monolayers of the test cells for 24 hours with a small (3-5mls) quantity of serumless medium.This medium was then removed and acidified for 1 hour at 37° C with 0.5M Glycine/HCl (pH 3.52) and then neutralised with NaOH.Small aliquots of this treated conditioned medium were then dropped onto the gel.The dishes were then left for 24 hours in the 37° C/5%CO_2 incubator after which the lysis was fixed with methanol and stained with Kenacid blue or Coomassie Blue,both prepared as described previously [2.7.2 b].

2.7.3. Antibody Tests

a) Immunoperoxidase Staining for Alpha-Fetoprotein (AFP).Cells were examined for the presence of AFP using immunohistochemistry involving indirect immunoperoxidase staining using a polyclonal antisera,raised in rabbit,to mouse AFP.

Cells were grown up on plastic "Thermanox" coverslips until a reasonable covering of cells was obtained (2-3 days).The cells were then fixed for 5 minutes in neat acetone and assayed for the presence of AFP.Preparations were first soaked in methanol to remove endogenous pigments and then,after rinsing in water for 5 minutes,they were treated with trypsin (0.1%)+$CaCl_2$(0.1%) in distilled water (pH 7.8),for 15 minutes at 37° C and were then assayed for AFP by the method for polyclonal antibodies, as shown in Table 2:5(a).

Table 2:5

METHOD FOR IMMUNOPEROXIDASE USING AFP

Summarises the protocol for staining slide preparations of tissue culture cells with Horse Raddish Peroxidase linked polyclonal antibodies (in this case AFP.

Immunoperoxidase Method for Polyclonal Antisera

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NT / ION	WASH	WASH	NSS 1:5	PRIMARY ANTIBODY AFP	WASH	NSS 1:5	SAR 1:20	WASH	NSS 1:5	PAP 1:100	WASH	DAB 5mg/5ml	WASH	HAEM COUNTER-STAIN
NT	WATER	TBS	TBS	1:5 NSS TBS	TBS	TBS	1:5 NSS TBS	TBS	TBS	1:5 NSS TBS	TBS	TRIS pH 7.6 + 0.1 ml 1% H_2O_2	WATER	
	5 MIN	5 MIN	10 MIN	30 MIN	5 MIN X 2	10 MIN	30 MIN	5 MIN X 2	10 MIN	30 MIN	5 MIN X 2	2-5 MINS	5 MIN	

ABBREVATIONS:

TBS - Tris buffered saline, pH 7.6
NSS - Normal swine serum
SAR - Swine anti-rabbit immunoglobulins
PAP - Peroxidase anti-peroxidase complex
DAB - diamino benzidine tetrohydrochloride

Notes

1) Primary antibody must be raised in rabbit
2) 0.01M Imidazole is added to the pH 7.6 Tris buffer for the DAB stage (Step 12)
3) TBS - Tris buffer is made up, and diluted x 10 with normal saline.

Adapted from: Immunopathology laboratory, Department of Pathology, Edinburgh University.

2.7.4. Electron Microscopy

For ultrastructural analysis by electron microscopy,cells were grown as monolayers on Permanox dishes (LUX).They were washed twice in PBSA and once in 0.1M Sodium Cacodylate.Monolayers were then fixed in situ with 3% (v/v) Glutaraldehyde in 0.1M Na Cacodylate and subjected to routine processing.

Sections were examined and photographed using a JEM-100S Electron microscope.

2.8. Photography

Photography was carried out in the lab using 35mm PanF film (64 ASA, Din 19) for black and white pictures and 35mm Ektachrome (Kodak) slide film for colour.All films were developed and printed in the department either by myself or by R.Simpson of the department technical staff.

Microscope slide pictures were taken using a Leitz Ortholux microscope using the objective magnification as indicated individually on the pictures,and the lower power photographs of growing cell cultures and embryos were taken using a Leitz Diavert inverted binocular microscope,both using a Leitz Vario-Orthomat camera.

CHAPTER 3

ISOLATION OF SOMATIC CELL HYBRID LINES FROM MEC+ AND MEC- EC CELL LINES : CHARACTERISATION AND COMPARISON WITH PARENT LINES

Previously it has been found that cell lines which are deficient in metabolic cooperation (such as R5/3OA) [64] are also unable to differentiate spontaneously to any great extent,although the use of chemicals such as Retinoic Acid can induce such changes in R5/3OA *in vitro*.

Cell lines like PSA4,which do differentiate can also cooperate and pass small metabolites from one cell to the next via their gap junctions,both to cells of the same line and to cells of other mec+ lines.

This raises the question of whether this observed link between differentiation and metabolic cooperation is of real significance,such that metabolic cooperation plays a vital role in one or more of the mechanisms of cellular differentiation,or whether this link is merely fortuitous and has a trivial explanation.

The construction of hybrid ec lines from a mec-,non-differentiating line (R5/3OA) and a mec+,differentiating line (PSA4) could therefore help to answer this problem.

This chapter describes the adaptation and modification of methods for the isolation of hybrid lines to enable the selection of hybrid ec lines derived from such cells and describes the characterisation of the lines obtained.

3.1. Establishment of a Method for Producing Viable EC Cell Hybrid Lines

This section describes preliminary fusogen experiments on co-cultures grown on STO feeder cells after Rosenstraus et al [135].These conditions were found to be unsatisfactory for the isolation of hybrid cell lines from PSA4 and R5/3OA using HAT and Ouabain medium because as expected the STO feeder cells were able to "rescue" the PSA4 cells from Ouabain toxicity by means of metabolic cooperation as evidenced by the presence of colonies of these cells visible in control dishes.

Recently [154,155] it had been found that feeder dependent cell lines such as PSA4 could be cultured,without differentiation,in feeder free culture if they were grown in medium conditioned by STO cells.This section reports preliminary experiments on STO conditioned medium and the modifications made to improve the effectivity of this medium as a substitute for STO feeder cells in monolayer cultures and also describes the adjustments made to the fusion protocol and selective medium which were necessary in order to isolate and grow up viable hybrid lines from the two parental ec cell lines PSA4 and R5/30A.

3.1.1. STO Conditioned Medium

Plating efficiency tests of the differentiating ec cell line PSA4 were carried out in order to confirm observations by T.Smith [154,155] that,by conditioning medium on STO cells one could overcome the feeder dependance of differentiating ec cell lines such as PSA4.

It was found that the optimum time for incubation of medium was 7 days [figure 3:1a] on feeder layers [section 2.3.3],in contrast to the 24 hours incubation on live STO cells recommended by T.Smith which was not found to be very satisfactory for these cells.This conclusion was based on both colony counts and morphological observation of the Leishmans' stained dishes of the plating efficiency experiments.As shown in Figure 3:1 colony counts were both greatest in number when grown in medium which had been conditioned on STO feeder layers for 7 days and also showed fewer apparently differentiated cells and colonies than were observed in cells and colonies of the same line (PSA4) grown on gelatin in medium conditioned on STO feeder cells for shorter (or Longer) periods of time or in medium not conditioned at all.

It was also found that "live" STO cells did not condition medium as effectively as STO feeder cells [figure 3: 1b] although this was only done using medium conditioned for 3-4 days.Medium could not be conditioned with growing STO cells for longer than this period because the monolayer culture reached confluence after 3-4 days and the medium became acidic.The fibroblasts (STO) could not grow under these conditions and so required subculturing.

Although colony numbers in plating efficiency experiments were as high or slightly higher [figure 3:Ib] as those obtained with medium conditioned for 7

Figure 3:1

PLATING EFFICIENCY OF PSA4 CELLS IN STO FIBROBLAST CONDITIONED MEDIUM

a) The effect of plating PSA4 cells in STO feeder conditioned medium incubated for varying lengths of time with the conditioning cells.

The effect of plating cells on gelatin - treated dishes (solid line) and on ordinary untreated dishes (dotted line) are also shown.

▼ -Gelatin treated tissue culture dish
▲ -Untreated tissue culture dish

b) The plating efficiency of PSA4 cells grown on STO feeder cells (+sto),on gelatin in medium conditioned with STO feeder cells (c+) and on gelatin in medium conditioned with live STO cells (Csto).

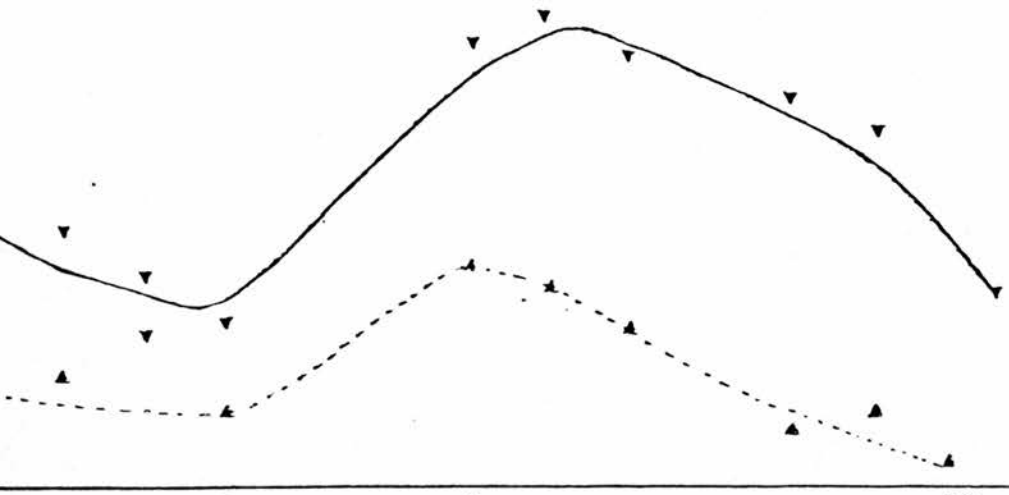
■ -STO feeder cells
● -STO conditioned medium (live cells)
▲- Feeder cell (STO) conditioned medium

Results are expressed as the number of colonies derived after incubation of dishes originally plated out at a density of 3000 cells per dish.

notes:

i) All conditioned medium was used at 100% concentration
ii) Conditioned medium used in 3:1b was supplemented with glutamine,10%NCS and B-Mercaptoethanol.Conditioned medium used in 3:1a was not supplemented in this way.

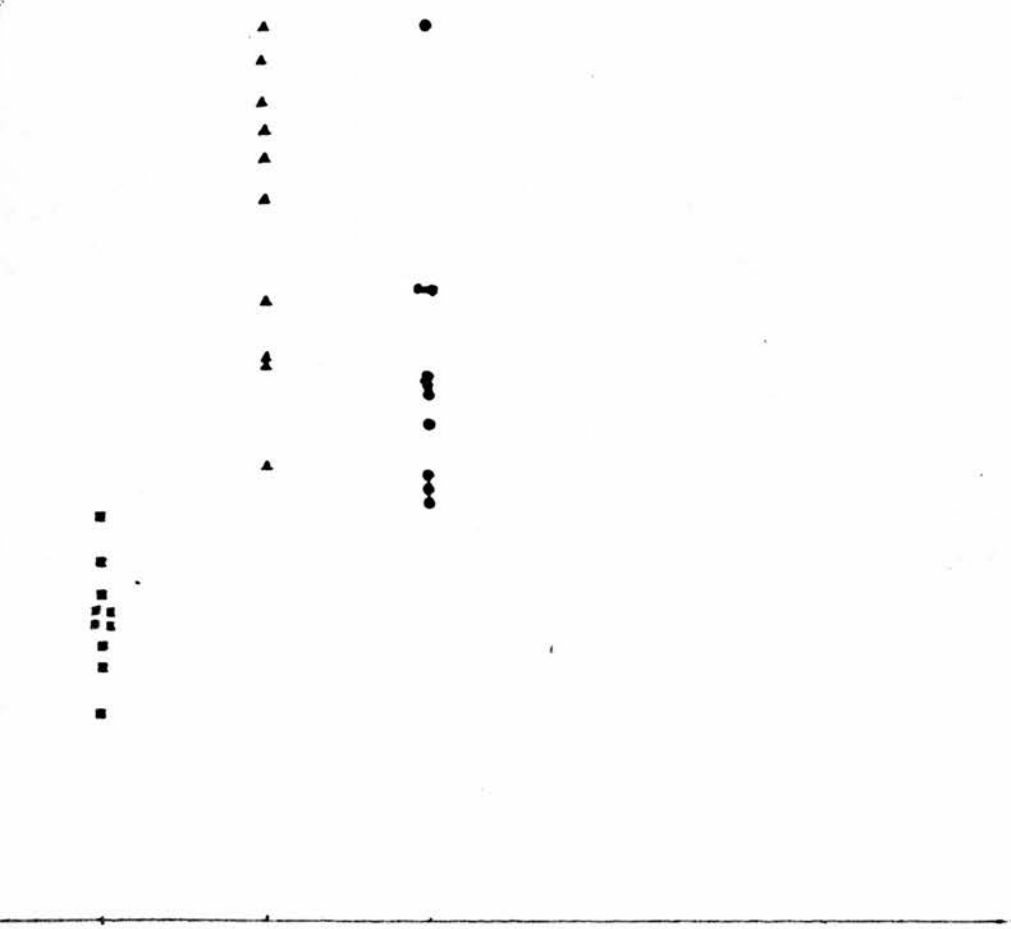
PLATING EFFICIENCY(%age)



3 6 9 12

LENGTH OF CONDTION
(days)

NUMBER OF COLONIES



+sto C+ Csto

days on STO feeder cells,the range of colony counts in different dishes was larger and the colony morphology was much more variable,so that there appeared to be less control of differentiation using live STO conditioned medium when compared with medium conditioned 7 days on STO feeder cells.It was,in any case,more convenient and easier to standardise medium conditioned on feeder layers because Mitomycin-C treated cells do not increase in number and were always plated out at the same density (10^6 cells per $25cm^2$).To further improve the consistency of the conditioning the same volume of medium and size of culture bottle (70 ml per 75 cm^2 bottle) was always used and feeder layers were only used once for conditioning medium.

Because medium was left on the feeder layers for 1 week it was found that results were also better and more consistent if conditioned medium was supplemented with Gln and with FCS (5-10%) which are the least stable components of the medium.B-mercaptoethanol was also added (10^{-14}M) since this had been reported to prevent differentiation of certain cells in feeder cell free conditions [135].

3.1.2. Use of PEG 6000 to Fuse EC Cells

Table 3:1 summarises all the methods tried and tested during the development of the final method which resulted in the isolation of hybrid lines from the two ec cell lines PSA4 and R5/3OA.

Hybrid isolation experiments were first attempted using a procedure reported by Rosenstraus et al [135] which employed a fusion protocol [2.3.2i] developed by Pontecorvo [128] and modified by Hales [53].This procedure is described in the methods chapter [section 2.3.6].

Coverslip preparations set up in parallel to monitor these experiments show [Table 3:2a] that Binucleate cells were obtained using this method and this evidence was supported by the appearance of binucleate cells in the dishes of the experiment which were quite clearly visible by phase contrast microscopy.However these cells disappeared after the first 2-3 days in culture and no colonies appeared in any of the dishes , with the exception of those plated out onto feeder layers [see 3.1.3].

Table 3:1

SUMMARY TABLE OF FUSION AND POST-FUSION PROTOCOLS

The table summarises the various modifications to fusion procedure and post-fusion treatment employed in the development of a method for the isolation of viable hybrids from the ec cell lines PSA4 and R5/3OA.

Results are listed in column five.

Method	Description of Fusion Procedure	Description of Post-Fusion Procedure	References	Remarks, Results and Problems
1	PEG 6000 in serial dilution from 1+1 (w/v) to 1+15 (w/v) PEG 6000 and CM(X). Treatment for 1 minute with initial 1+1 dilution.	Monolayers trypsinised after 2 hours. Plated onto STO feeder layers in 90mm dishes. fed with HAT + Oua medium after 18-24 hours. Coverslips left overnight then stained with Leishmans.	1. Rosenstraus, M.J. Balint, +Levine (1980) Som.Cell Gen.6 555-565. 2. Pontecorvo, G. (1975) Som.Cell Gen.1 377- 3. Pontecorvo, G. Riddle, P.N. & Hales, A. (1977) Nature 265 257-258.	Feeder rescue of parental cell from the selection procedure. N heterokaryon survival after th first 2-3 days. 3 experiments.
2	As for 1. Ratio of R5/3OA to PSA4 increased to 5:1.	As for 1 except that the cells were plated out on to Gel treated dishes rather than onto STO feeder cells.		No heterokaryon survival after the first 2-3 days. some feeder rescue due to residual feeders No lines selected. 1 experiment.
3	As for 2.	As for 2 except that STO feeder conditioned medium was used for the initial plating out and as a base for the HAT+Oua medium.	4. Smith, T.A. & Hooper, M.L. (1983) Expt.Cell Res. 145 modified 458-462.	Slightly better cell survival but still no heterokaryon survival after the first few days. No lines selected. 9 experiments.
4	PEG 6000 dissolved 50:50 in CM(X)+10% DMSO. Treatment for 2 mins at room temperature. 3x wash with CM(X)+DMSO 1x wash with CM(10)FCS. Ratio of R5/3OA to PSA4:9:1 2x10 cells per bottle.	As for 3. Gln and 5-10% FCS supplements to the filtered STO conditioned medium.	5. Oshima, R.G., McKerrow, J. & Cox, D. (1981) J.Cell Physiol. 109 195-204.	No cell lines selected. No noticible improvement in heterokaryon survival. 2 experiments.
5	Monolayers Prepared as for 4. Treated with 50% PEG 1000 diluted into HEPES (0.15M) buffer at PH 7.55.1 Minute at room temperature washed 3x with Calcium free medium.	Cultured 1 week before trypsinisation, using enriched medium for the first 24 hours, then including HAT + Oua in this medium for selection.	6. Klebe, R.J. Mancuso, M.G. (1981) Som.Cell Gen. 7 473-488. 7. Evans, M.J and Kaufman, M.H. (1981) Nature 292 154-156.	First experiment produced 1 cell line with a karyotype of of over 100 (PR1). A second experiment (using several co-culture flasks) isolated another 4 cell lines: PR2, PR3, PR4 and PR5. 2 experiments.

3.1.3. Feeder Rescue and Use of STO Conditioned Medium

It was found that if the fusion treated cells were plated out onto STO feeder cells,as suggested by Rosenstraus et al,[135] then the PSA4 cells were rescued from ouabain toxicity by metabolic cooperation with the feeder cells.The PSA4 parental cells therefore survived the selective medium treatment thus invalidating the selection procedure.This was shown by the presence of surviving cells in PSA4 control dishes as well as by the PSA4 morphology of the surviving cells in coculture dishes.

Cells were for this reason plated out onto gelatin only in further experiments to avoid this complication [Table 3:1, 2].

Further modification [Table 3:1, 3] using STO conditioned medium [section 3.1.1] enabled better survival of the feeder dependent cells and therefore was expected to improve the survival rate of heterokaryons.However no cell lines were isolated from any of the 9 experiments set up in this way since,despite the observation of heterokaryons over the first 2-4 days after fusogen treatment , both in living culture and on coverslip preparations [3.1.4] as described for 2,none of these survived the selection any further and so no colonies grew up.

3.1.4. Coverslip Counts of PEG 6000 Treated Co-cultures

The coverslips were scored by counting nuclei and cells to give the Nucleus:Cell ratio (N/C) and also by counting the number of binucleate cells which gave the overall percentage of binucleate cells.The results of three separate experiments were aggregated to give the results shown in Table 3.2a. As was expected the control slides had no multinucleate cells and so gave an N/C ratio of 1.0,and therefore 0% of Binucleate cells.In contrast to this it was found that the N/C ratio of PEG 6000 treated co-culture cells plated onto gelatin was 1.43 which indicates a considerable amount of cell fusion,7.25% of the cells counted were binucleate.Of the cells plated out onto STO feeder cells it was found that slightly more (7.53%) were binucleate but that the N/C ratio was reduced to 1.19 indicating that the overall fusion rate was lower.This difference and gives a total mean % of binucleate cells of 7.39% + 0.14 and a mean N/C ration of 1.33 + 0.12.

Table 3:2

ANALYSIS OF BI- AND MULIT-NUCLEATE CELLS PRESENT IN COVERSLIP PREPARATIONS OF FUSOGEN TREATED AND CONTROL CULTURES OF THE EC CELL LINES PSA4 AND R5/3OA

Plastic 'Thermanox' coverslips were set up containing samples of co-cultures of PSA4 and R5/3 OA which had been stained with Leishman's stain.

a) Results of counting the number of binucleate cells and the nucleus cell (N C) ratio of cells treated with PEG 6000 (PEG) in comparison to control slides(c) not treated with fusogen.

b) Results of counting binucleate cells and N C ratio of cells treated with PEG 1000 in HEPES buffer (PEG) compared with controls (C).

Key:

(G) - cells plated onto gelatin covered coverslips

(F) - cells plated onto STO feeder layers

(T) - pooled data from (G) and (F) counts

%bi - percentage of binucleate cells of those counted

P - PSA4 cells
R - R5/3OA cells
P+R - co-culture of PSA4 and R5/3OA cells

a)

		N	C	Binucleates	N/C	%Bi
PEG	P+R(G)	573	400	29	1.43	7.25
	P+R(F)	331	279	21	1.19	7.53
	P+R(T)	904	679	50	1.33	7.39
					(+0.12)	(+0.14)
C	P+R(T)	200	200	0	1.00	0

b)

		N	C	Binucleates	N/C	%Bi
PEG	P+R(G)	348	290	42	1.20	14.4
	P+R(F)	288	235	44	1.23	18.7
	P+R(T)	636	525	86	1.22	16.4
					(+0.02)	(+2.15)
C	P+R(G)	197	195	2	1.01	1.0
	P+R(F)	136	136	0	1.00	0
	P+R(T)	333	331	2	1.01	0.6
					(+o.005)	(+0.5)

All slides were counted under oil immersion with the x100 lens.

3.1.5. Fusion of EC Cells Using PEG 6000 (50%

and DMSO (10%))

A further two experiments attempting to isolate a hybrid line from the ec lines PSA4 and R5/3OA used a fusion mixture described by Oshima et al [123] see Method 3 [2.3.6].However although,as before,binucleate cells were observed in the selection for the first few days,no colonies survived and therefore no cell lines were isolated.

3.1.6. Development of Enriched Medium

Reports by Evans et al [person.comm.] indicated that it was possible to grow up cell lines from mouse blastocyst embryos with the aid of medium that had been enriched by increasing the serum content to 20% and by adding B-mercaptoethanol (10^{-4}M).It was therefore thought possible that the problem of heterokaryon death in hybrid selection procedures [sections 3.1.2,3.1.4. and 3.1.5.] might be overcome by enriching the medium and so improving the culture conditions sufficiently to allow some of the heterokaryons to undergo their first cell division and produce hybrid cells which could then be grown into permanent cell lines.

Figure 3:3 shows the effect of increasing the percentage of FCS,at the expense of NCS percentage,in culture medium with a total serum content of 20%.It shows clearly that the plating efficiency of PSA4 cells is positively correlated with the percentage of FCS present in the media,despite the fact that these cells are routinely grown in CM containing 10% NCS only.

The two control dishes,one showing plating efficiency in 10% FCS only and one in 10% NCS only fall onto the growth curve,respectively in the positions of 10% FCS and 0% FCS.These results suggest that new born calf serum has no additional effect on cell growth in the presence of foetal calf serum.

Figure 3:2

THE EFFECT OF INCREASING FOETAL CALF SERUM IN CELL CULTURE MEDIUM,ON THE PLATING EFFICIENCY OF PSA4 CELLS

Showing the effect of increasing the percentage of foetal calf serum (FCS) in medium with a total serum content of 20%.

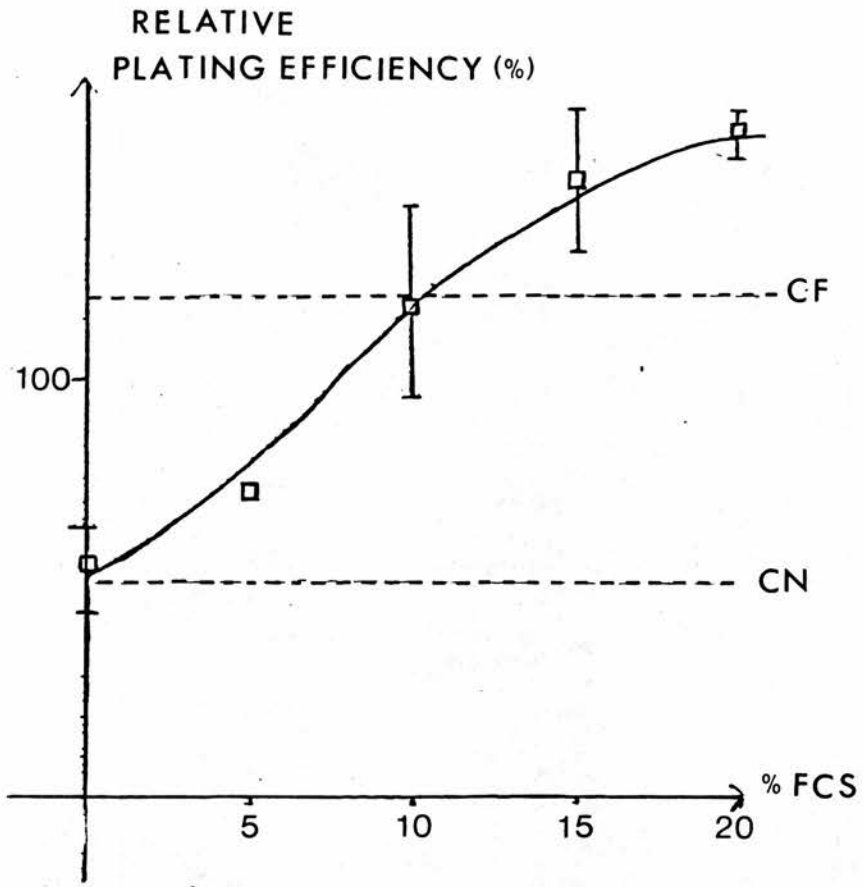
Two control points CF and CN illustrate the plating efficiency of the same cell line when incubated with medium containing 10%FCS (CF) and 10%NCS (CN) respectively.

CN - Plating efficiency in 10% newborn calf serum

CF - Plating efficiency in 10% foetal calf serum

□ - Plating efficiency of cells in 20% calf serum

Relative Plating Efficiency : Percentage of colony counts in relation to control count


RELATIVE
PLATING EFFICIENCY (%)
100
CF
CN
% FCS
5
10
15
20

3.1.7. Coverslip Counts of Cells Treated With PEG 1000 in HEPES Buffer

Coverslips of PSA4 and R5/3OA co-cultures were treated with the PEG 1000 in HEPES buffer fusogen and then stained and mounted on slides the following day [2.3.6]. Control counts gave an N/C ratio of 1.0 and 1.01 respectively for controls (not treated with PEG) plated onto feeder layers and those plated onto gelatin.The N/C ratio was above 1.0 for the gelatin control because the counts included two binucleate cells (1%) which had apparently occurred naturally.The N/C ratios of the PEG treated cells were 1.2 for the ones plated onto gelatin and 1.23 for those plated onto STO feeder cells.The mean total of percentage binucleate cells counted was 16.4% + 2.15 this figure includes 14.4% of binucleates in the Gel plated co-cultures and 18.7% in the STO feeder plated Co-cultures.

3.1.8. Method for Isolating hybrid Cell Lines from the EC Cell lines PSA4 and R5/3OA

The method [Method 4,] finally employed to sucessfully isolate hybrid cell lines from the two ec cell lines R5/3OA and PSA4 incorporated three main alterations to the original method [see Table 3:1,£5].These modifications,listed below, were designed to protect the heterokaryons over the first few days in culture and to enhance their chances of survival into hybrid cell lines.

i) The use of enriched,STO conditioned medium,as described in the results section on enriched medium [3.1.6,for method see section 2.3.4] which had been shown to enhance ec cell survival and cell growth.

ii) The use of PEG 1000 in HEPES (0.15M) buffer [section 3.1.7] which was shown to give an increased proportion of Bi-nucleate cell formation when compared to the PEG 6000 treatment and also has been reported to give improved fused cell survival [79].

iii) After treatment with the fusogen the monolayer co-cultures were not trypsinised as in previous experiments, but were allowed to "recover" for 7 days in their original monolayer,during this time they were fed with enriched selective medium [2.3.4].After this time the monolayers were plated out onto gelatin and colonies were grown up in the selective medium which was

enriched with 20% FCS and with 10^{-4} B-Mercaptoethanol.

The use of these modifications yielded colonies of cells growing up in the HAT and Oua Supplemented medium.The first of these experiments produced the cell line PR1 which unfortunately was lost through contamination before permanant stocks could be made.It was however karyotyped and the chromosome count of over 100 with two metacentric marker chromosomes [3.3.I] and its survival of the selection procedure indicated that this was probably a hybrid cell line.

The second experiment using the final modification [summarised in Table 3:1,£5] yielded four independent cell lines PR2,PR3,PR4 and PR5.This was achieved by treating several separate bottles of monolayer co-cultures with fusogen and isolating one colony only from each of them.The line PR3/4 arose from an accidental contamination of the line PR4 with PR3 cells and the line PR2 was lost through bacterial contamination.

However permanent stocks of the lines PR3,PR3/4,and PR5 were made and the remainder of this chapter is concerned with the characterisation of these putative hybrid cell lines and with a discussion of their properties with particular reference to the differentiation behaviour and metabolic cooperation of these cells.

3.2. Morphological Characteristics and Growth Requirement of Cell Lines Isolated by PEG 1000 Fusogen Treatment

3.2.1. Morphology Using Phase Contrast Microscopy

All of the cell lines isolated by the PEG 1000 fusion method resembled ec cells in their morphology as seen by the phase contrast microscope [Figure 3:4].They were all large epithelioid cells with the nucleus filling the major part of the cell and several prominent nucleoli.When seeded onto STO feeder cells they grew tightly packed together in clumps resembling the growth of the feeder dependent line PSA4 more closely than the R5/3OA cell line,although it is acknowledged that the distribution of the STO feeder cells on the culture surface may have had some influence on the colony morphology of the cells growing on them.

Figure 3:3

THE LIGHT MICROSCOPE MORPHOLOGY OF HYBRID LINES
PR3 AND PR3/4 AND THE DIPLOID LINE PR5

Shows the morphology of monolayer cultures of the cell lines PR3,PR3/4 and PR5 when viewed by phase contrast microscopy.

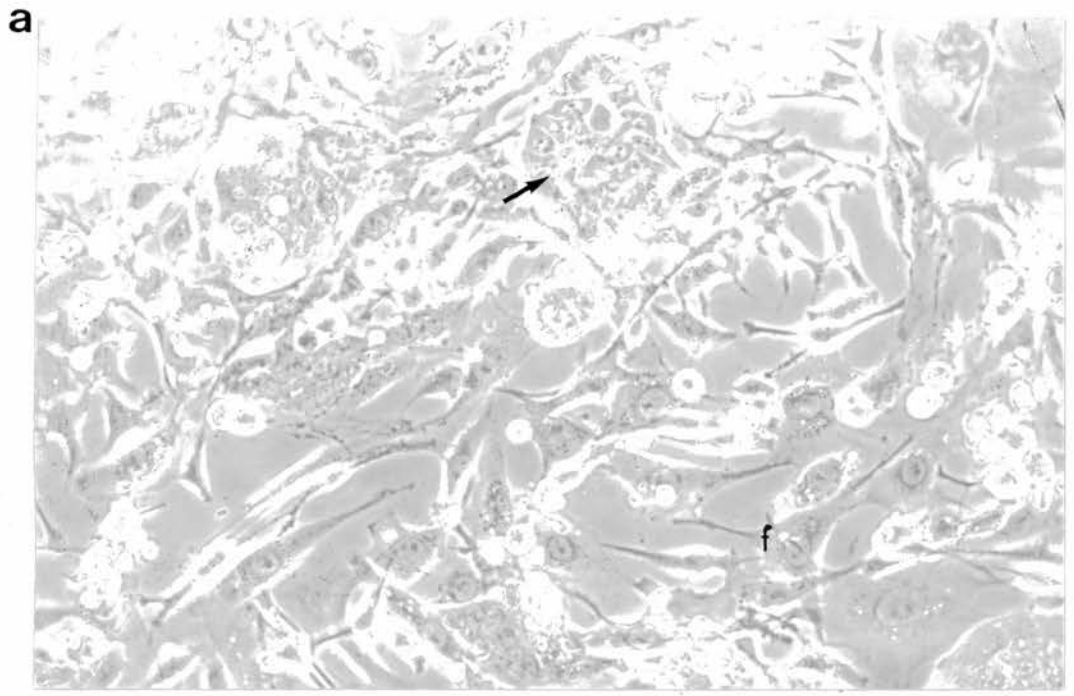
a) Monolayer culture of the hybrid line PR3 growing on an STO feeder layer (f).Arrow indicates a colony of PR3 ec cells.

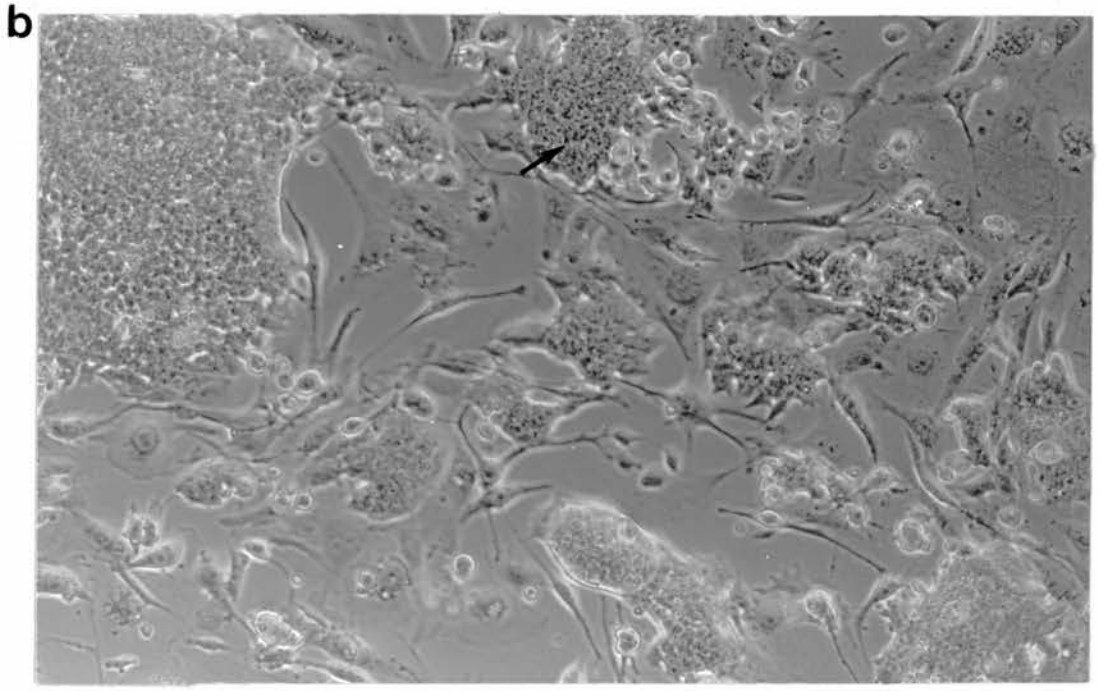
b) Monolayer culture of PR5 cells growing on an STO fibroblast feeder layer.Cells are growing in characteristic tightly packed colonies resembling those of the ec line PSA4.Arrow indicates a PR5 colony.

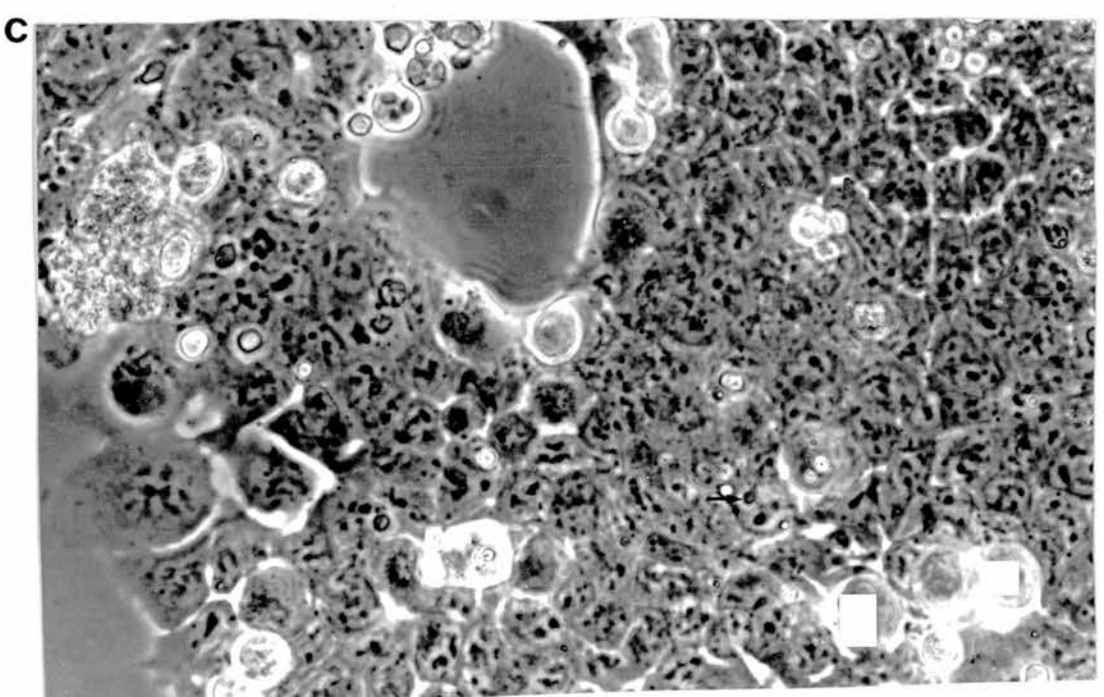
Both of **a)** and **b)** were taken at low power (x10 Objective) and have an overall magnification of 320.

c) Monolayer culture of PR3/4 growing on an STO fibroblast feeder layer and taken at higher magnification (x32 Objective) giving an overall magnification of 1024.

Arrow indicates the characteristic,darkly stained nucleoli present in all three of the ec lines PR3, PR3/4 and PR5.


a
f


b


c

However,the two lines PR3 and PR3/4 both seemed to grow less tightly packed together than did the lines PR5 and PSA4 which were indistinguishable by their light microscope morphology in monolayer culture.It was also observed that the number of nucleoli per cell nucleus had increased from 2-4 in the PSA4 and PR5 cell lines to 4-6,and ocasionally as many as 8-10,in the PR3 and PR3/4 cell lines.

When these cell lines (PR3,PR3/4,PR5,PSA4) were grown in the absence of feeder cells,on gelatin in STO feeder conditioned medium [2.3.3],they had a similar appearance to that described for monolayer cultures growing on STO feeders,except that some of the colonies started to produce differentiated cells.This phenomenon is discussed in greater detail in Chapter 5 and section 3.5.1. of this chapter.

3.2.2. Feeder Dependence and the Effects of Altering Serum Concentration

When the cell lines PR5,PR3/4 and PR3 were plated out into 10% serum (FCS) they all displayed better plating efficiencies when plated onto STO feeder layers than when plated onto gelatin alone [Figure 3:5].This result is in comparison with the feeder dependent cell line PSA4 which grew well on STO feeder layers at 10% FCS and poorly on gelatin at this serum concentration.In contrast the cell line R5/3OA grew well in 10% FCS whether it was plated onto gelatin or onto STO feeder layers.

The improvement in plating efficiency due to STO feeder layers was negligible for the line PR3/4 however which grew well on both gelatin and feeder cells and therefore more closely resembles R5/3OA in this respect.The line PR5 however did not grow very well in 10% FCS at all although small numbers of colonies were present in both gelatin and STO feeder dishes,and the numbers of colonies per dish was substantialy increased in the latter.

The growth of all of these cell lines was improved when they were grown in 20% serum (FCS),the most dramatic change being that of PR3 whose growth became feeder independent at this concentration of FCS despite its almost complete lack of growth on gelatin at the lower level of serum.The line PR5 still did not grow very well when compared to the other lines even in 20% serum and so it was routinely maintained on STO feeder cells in medium containing this level of serum .The lines PR3 and PR3/4 were subsequently

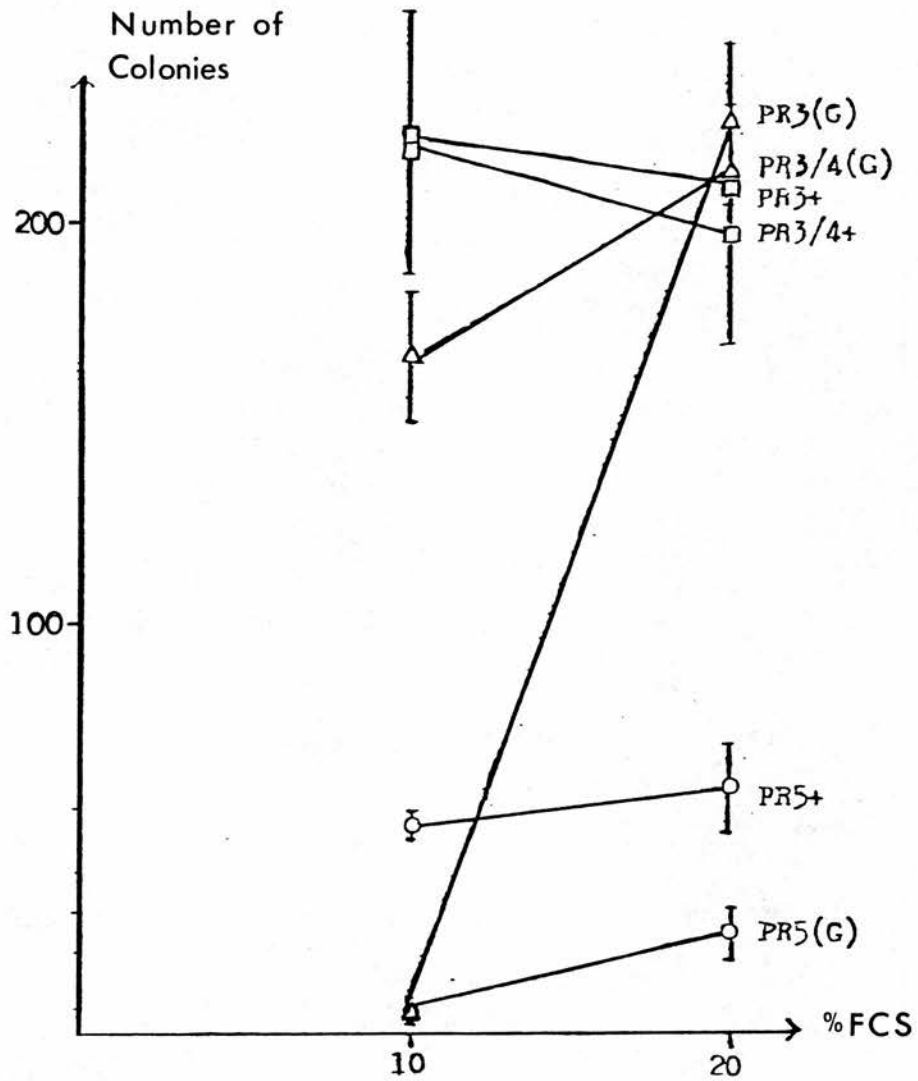
Figure 3:4

THE RELATIONSHIP BETWEEN FEEDER DEPENDENCE AND CONCENTRATION OF FOETAL CALF SERUM IN DERIVATIVES OF PSA4 AND R5/30A CELL FUSION EXPERIMENTS

Shows the effect of increasing FCS Concentration on the number of colonies of the lines PR3,PR3/4 and PR5 on STO feeder cells and on gelatin treated tissue culture dishes in the absence of either feeder cells or conditioned medium.

△ - PR3
□ - PR3 4
○ - Pr5
(G) - gelatin
\+ - feeders

Plating efficiency is expressed as the number of colonies surviving after incubation of dishes plated initially at a density of 3000 cells per dish.


Number of Colonies
200
100
PR3(G)
PR3/4(G)
PR3+
PR3/4+
PR5+
PR5(G)
%FCS
10
20

routinely grown in 10% FCS on STO feeder layers since this represented a more economical compromise than plating these cell lines out in 20% serum on gelatinised dishes.Also some differentiation of all cell lines was observed when they were plated onto gelatin even when in 20% FCS.This was not observed in any of the lines when they were plated onto STO feeder cells.

3.2.3. Plating Efficiency of putative Hybrid Cell Lines in HAT and Ouabain

The PR lines were tested for their ability to grow in HAT and Ouabain separately and in combination,as a further test of their hybrid origin as well as simply attempting to show that cell lines selected in medium containing both HAT and Ouabain were resistant to both of these substances. Unfortunately the results obtained with these plating tests were not as unambiguous as was expected and possible reasons for this are explored in the discussion section [3.6].

As expected neither PSA4 nor R5/3OA would grow in the combined HAT and Ouabain selective medium.R5/3OA would not grow in HAT medium but exhibited resistance to Ouabain,although in some experiments the survival of R5/3OA cells in this medium was minimal [Figure 3:5a].PSA4 grew well in HAT medium although the plating efficiency as compared with the control dishes varied quite widely from one experiment to another [section 3.6].The low survival rate of PSA4 in Ouabain medium in some experiments was probably due to rescue of the ec cells by metabolic cooperation with residual Ouabain resistant STO feeder cells [section 3.1.2].

The three lines PR3,PR3/4 and PR5 all exhibited a wide range of comparative plating efficiencies in all three types of media,although all three lines showed resistance to both HAT and Ouabain.These results are therefore consistent with their being selected from the two ec lines PSA4 and R5/3OA in medium containing both HAT and Ouabain.

3.2.4. Plating Efficiency of Putative Hybrid Cell Lines in 6- Thioguanine

The plating efficiency of the two parent lines PSA4 and R5/3OA in various concentrations of 6-Tg [Figure 3:5b] showed that the line PSA4 was incapable of growth in any concentration of 6-Tg but that the line R5/3OA which is

Figure 3:5

PLATING EFFICIENCY OF PSA4,R5/3OA AND THEIR DERIVATIVES
PR3,PR3 4 AND PR5 IN HAT,OUABAIN AND 6-THIOGUANINE

a) Collated results (8 experiments) of plating efficiency experiments of putative hybrid and their parent lines in medium containing HAT,Oua or both HAT and Ouabain.Cells were plated out at cloning density (1000 cells per dish) in STO conditioned medium,and the colonies stained with Leishman's after 5-6 days incubation,before being counted using a binocular microscope.

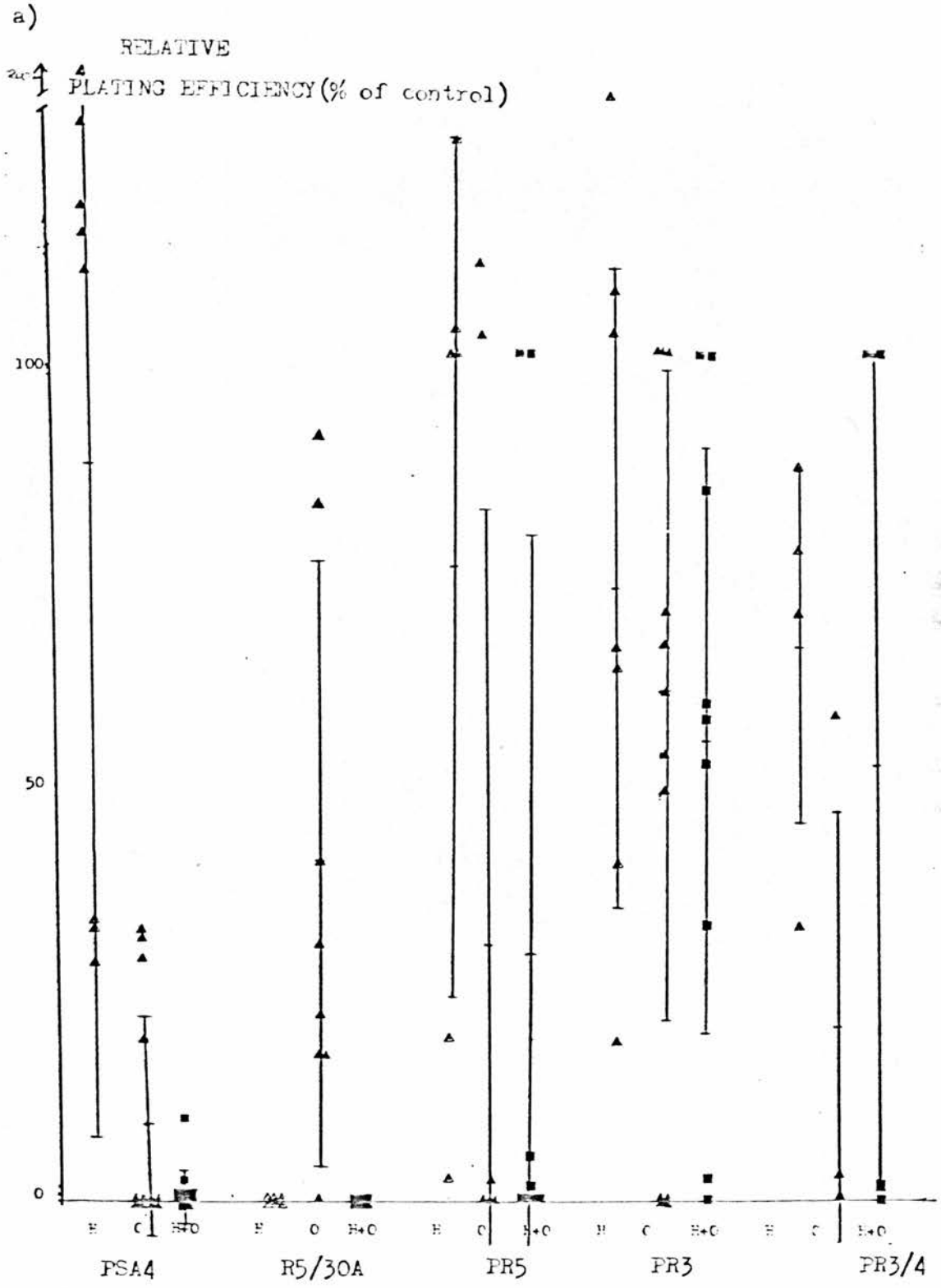
△ - HAT
▲ - Oua
■ - HAT + Oua

note: i) All cell lines except R5/3OA (which does not require feeder cells) were adapted to conditioned medium for one passage to attempt to remove residual feeder cells which could interfer with the toxicity tests by 'rescuing' sensitive cells by the mechanism of metabolic cooperation.

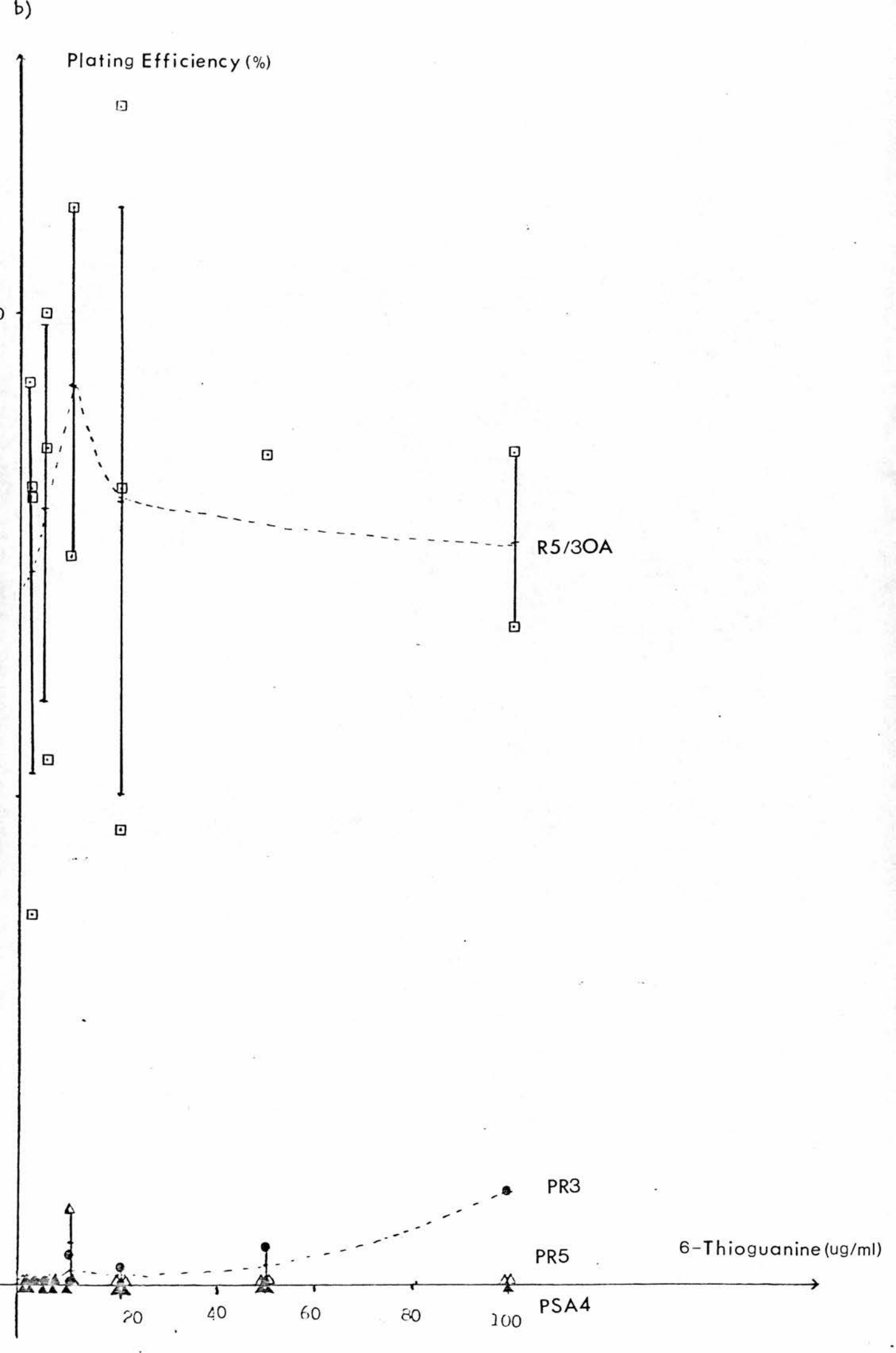
b) Plating efficiency of R5/3OA,PR3,PR5 and PSA4 cells in 6-Thio guanine shows that the line R5/3OA is resistant to this drug but that PSA4 and the hybrid lines PR3 and PR5 are unable to grow in medium containing thioguanine.

□ - R5/3OA
● - PR3
△ - PR5
▲ - PSA4

note: ii) Bars in both 3:5a and 3:5b represent the mean + 1SD (Standard Deviation of the Mean)


a)
RELATIVE
PLATING EFFICIENCY (% of control)
100
50
0
H
C
H+C
PSA4
R5/30A
PR5
PR3
PR3/4

b)


Plating Efficiency (%)
R5/30A
PR3
PR5
PSA4
6-Thioguanine (ug/ml)
20
40
60
80
100

HPRT- would grow well in concentrations of 6-Tg of up to at least 100µg/ml.

Neither of the two lines PR5 and PR3/4 which were derived from these cells are able to grow well in any concentration of 6-Tg.PR5 is comparable with PSA4 in this respect in that it did not grow at all in 6-Tg.The majority of dishes of PR3 cells showed no survival of cells in 6-Tg indicating that this line also was thioguanine sensitive [Figure 3:5b].

3.3. Karyotyping of PR Lines

The parental lines PSA4 and R5/3OA had been previously karyotyped [59,153] and had been shown to have karyotypes which were respectively approximately diploid (PSA4) and Tetraploid (R5/3OA) [see Table 2:1,methods 2.1.1].In addition to this the line R5/3OA had been shown to have two metacentric marker chromosomes.These results have been confirmed by myself [Figure 3:6] and the karyotypes of these two lines are described in the following sections together with those of the lines PR1,PR2,PR3,PR3/4 and PR5,all of which have been derived from cocultures of these two lines and therefore,if they are PSA4:R5/3OA hybrids,are expected to have approximately hexaploid karyotypes (120 chromosomes) including the two Marker chromosomes from the R5/3OA line.

3.3.1. Chromosome Counts

The karyotypes of the putative hybrid lines were first assessed by counting the number of chromosomes per metaphase spread of drop slide preparations made from growing monolayer cultures of each line [section 2.4.1].Because the range of counts was quite large and this aspect of characterisation was considered to be a crucial part of hybrid line identification,at least 100 spreads were counted (rather than the usual 20), per cell line.This was unfortunately not possible,however,for the two lines PR1 and PR2 which were lost through contamination,but those counts which were available for these cell lines have been included in the results [Figure 3:6].

The results of these counts clearly identify the lines PR3 and PR3/4 as hybrids and show that the lines PR1 and PR2 were also probably hybrids.The two hybrid lines PR3/4 and PR3 have modal chromosome counts of 100 and 102

Figure 3:6

KARYOTYPE OF PSA4,R5/30A AND DERIVATIVE LINES PR3,PR3/4 AND PR5

Showing the results of counting metaphase spreads of (100 per line with the exception of PR1 and PR2 where this was not possible) colchicine treated preparations of monolayer cultures.

a) Summary table of statistical analysis of metaphase spread chromosome counts.Includes the number of metaphase spreads found in each line.

b) Histogram plot of counts

a)

Cell Line	Mode	Mean	SD	Range	Metacentrics	n
PSA4	40	38	3.9	23-82	0	99
R5/30A	68	73	7.7	52-100	2	101
PR1	110	nd	nd	102-119	nd	10
PR2	119	nd	nd	93-128	nd	30
PR3 t	102	92	27	35-158	2	114
a		102	14	56-158	2	95
b		38	2.3	35-42	0-2	19
PR3/4	100	97	18	59-165	1-2	99
PR5	38	36.7	4.9	19-62	0	100

notes:

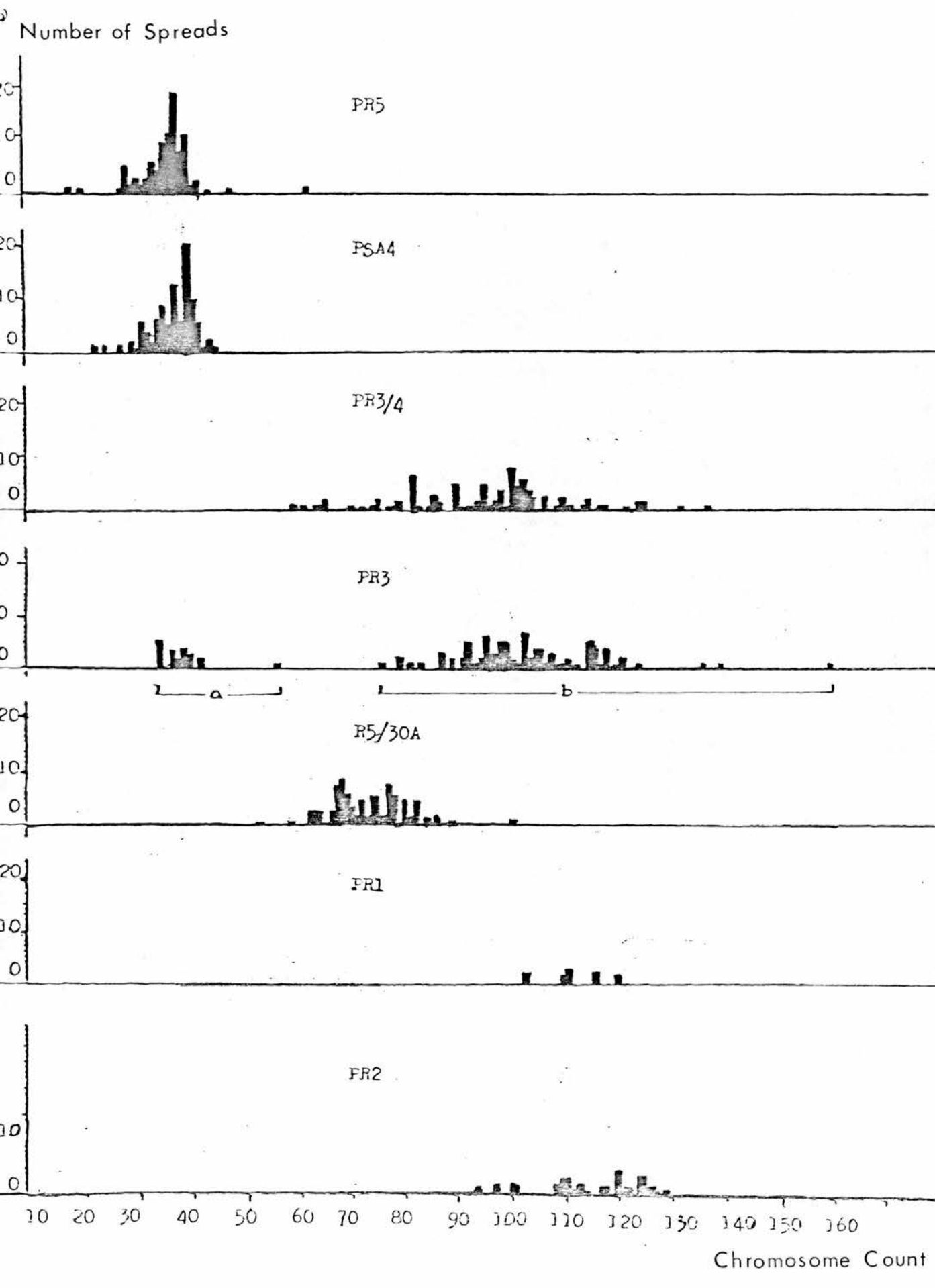
i) n is the number of metaphase spreads counted per line or per interline group.

ii) t is the analysis for the total number of metaphase spreads counted.

iii) a is an intraline group of metaphase spreads which fall below the value of 42 chromosmes per spread.

iv) b is the majority group of metaphase spreads counted for the line PR3 (see 3:7b) which fall between 56-158.

v) nd indicates that this data was not collected.


Number of Spreads
PR5
PSA4
PR3/4
PR3
a
b
R5/30A
PR1
PR2
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
Chromosome Count

respectively.These values are significantly greater than the modal values of the tetraploid line R5/3OA (mode of 68) and the diploid line PSA4 (mode of 40) and are,in fact,close to the sum of these two modes (108) which is the expected result from a hybrid derived from the fusion of a single R5/3OA cell with a single PSA4 cell.

Because the range of chromosome counts in these "hybrid" lines was quite large and the modal values not a significant proportion of the cells counted,the mean chromosome counts are also quoted and these are 97 and 103 respectively for the lines PR3/4 and PR3.These values also agree with the conclusion that both of these lines were derived from PSA4/R5/3OA fusion event(s).

The cell line PR5 (with a modal chromosome count of 38) was clearly not a hybrid line and its chromosome count profile closely resembles that of the line PSA4.This line (PR5) is therefore probably a ouabain resistant diploid derivative of PSA4 rather than a fusion product of PSA4 and R5/3OA.

3.3.2. **Metacentric Chromosomes**

The two metacentric chromosomes present in the line R5/3OA were identified in karyotypes of R5/3OA and were also found in the majority of metaphase spreads examined of both the hybrid lines PR3/4 and PR3 [Figure 3:7].These two marker chromosomes were also observed in karyotypes of the two lines PR1 and PR2.All of the chromosomes in cells examined of the lines PR5 and PSA4 were found to be acrocentric and the marker metacentric chromosome were not present [Figure 3:7].

3.4. **Metabolic Cooperation of Cell Lines using Uridine Transfer Analysis**

The PR lines (PR3,PR3/4) are thought to be derived from the fusion of the Mec+ line PSA4,whose cells are capable of transfering uridine nucleotides via gap junction mediation and the Mec- line R5/3OA which has a reduced ability in this respect and is therefore cooperation deficient.

These properties of the parental lines R5/3OA and PSA4 were confirmed in autoradiography experiments in which tritiated uridine transfer was analysed

using experiments in which donor and recipient were of the same cell line,nucleotide transfer of this type is conventionaly referred to as "homotypic".The cooperation properties of the two hybrid lines PR3 and PR3/4 were investigated and also those of the PSA4 derivative line PR5.

Of four experiments using tritiated uridine transfer,two [3:8c and 3:8d] involved incubation of donors with recipients for 3 hours while the remaining two were involved incubations of 4 hours each.The results of all of these experiments are detailed in combined tabular and histogram form in Figures 3:8a-3:8d.The results and means of all experiments are summarised in Table 3:5.

3.4.1. Metabolic Cooperation Properties of Hybrid Lines in Comparison to the Parent Lines

Metabolic cooperation of the hybrid (PR3,PR3/4),PSA4 derived (PR5) and parental lines (PSA4,R5/3OA) were measured by the analysis of tritiated uridine transfer in four separate experiments.All of these experiments were set up in duplicate and some in triplicate [see Figures 3:8a-3:8d].Two of these experiments [3:8c and 3:8d] are part of two separate large experiments involving cell lines reported in Chapters 4 and 5 as well as in this chapter.This allows the direct comparison of the results of uridine transfer experiments between all three chapters.These two experiments were designated 27/3 and 9/4 respectively and the remainder of the results of these two experiments can be found in Figures 4:11 and 5:4.

The results confirm that the parent lines PSA4 and R5/3OA are respectively mec+ and mec-.R5/3OA has an average T-NT/X value of between 1.08 and 1.5 and a range of percentage cooperation estimates of 63-83% (2 experiments).PSA4 on the other hand has T-NT/X values of between 5.5 and 20 and percentage cooperation estimates of 81-98%.

The PSA4 derivative line PR5 has been shown (3/4 experiments) to have a similar cooperation ability to that displayed by PSA4 with percentage cooperation estimates of 87.3-98 and T-NT/X values of 5.5-17.3.In the 4th experiment the cooperation ability of this line appeared to be rather lower with a mean T-NT/X value of 3.3 and mean percentage cooperation estimate of 83%.In this same experiment the mean percentage cooperation estimate for PSA4 was 96 and the mean T-NT/X value was 10.5.

Figure 3:7

ILLUSTRATION OF THE KARYOTYPE OF THE HYBRID LINES PR3 AND PR3/4 AND THE DIPLOID DERIVATIVE LINE PR5

a) Trypsin banded metaphase spread of PR3 with a chromosome count of 109.Two metacentric marker chromsomes characteristic of these cells,are indicated by arrows.

Print magnification was 4 - Overall magnification = 3200

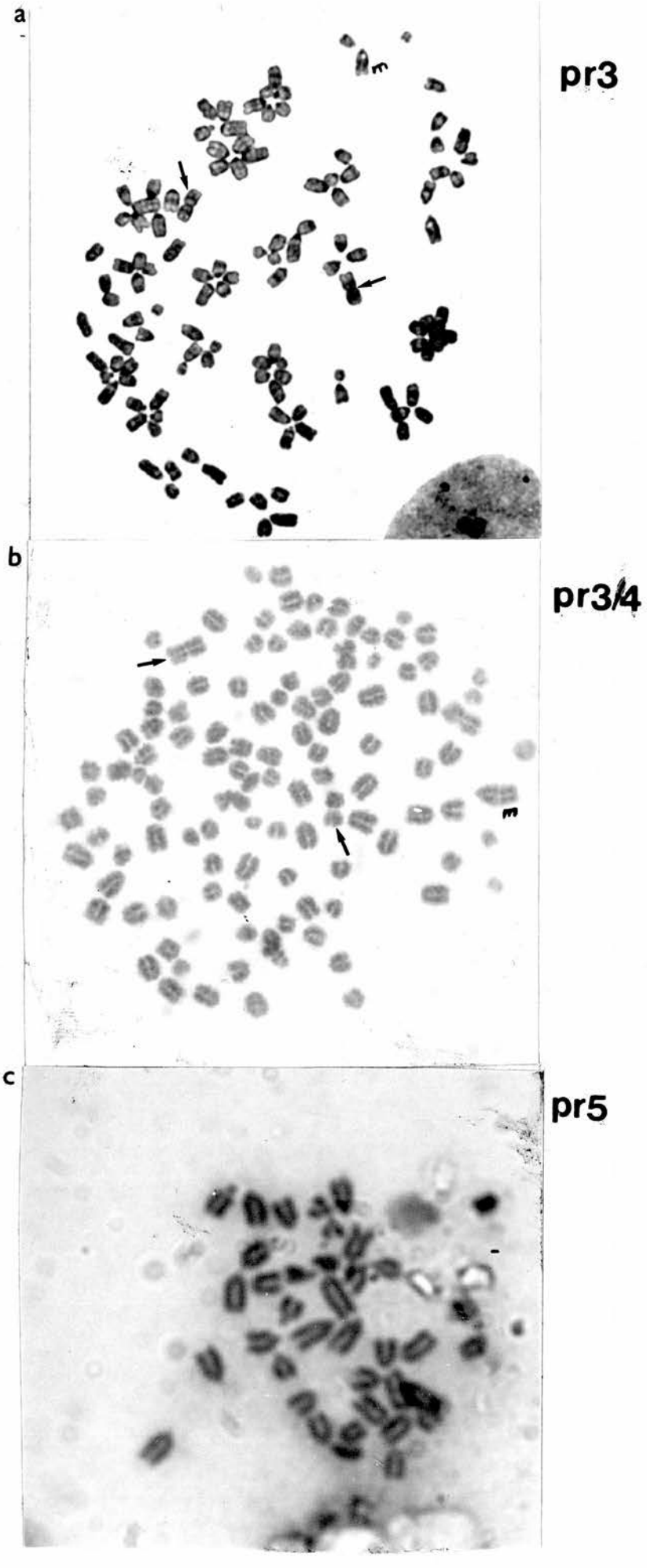
b) Metaphase spread of PR3/4 with a chromosome count of 117 and two metacentric marker chromosomes indicated by arrows.

print magnification was 4 - Overall magnification = 3200

c) Metaphase spread of PR5 with a chromosome count of 38.

Print magnification was 8 - Overall magnification = 6400

All three preparations of metaphase spreads were stained with Leishman's stain diluted 1:3 in Gurr's buffer at pH of 6.8.


a
pr3
b
pr3/4
c
pr5

Figures 3:8

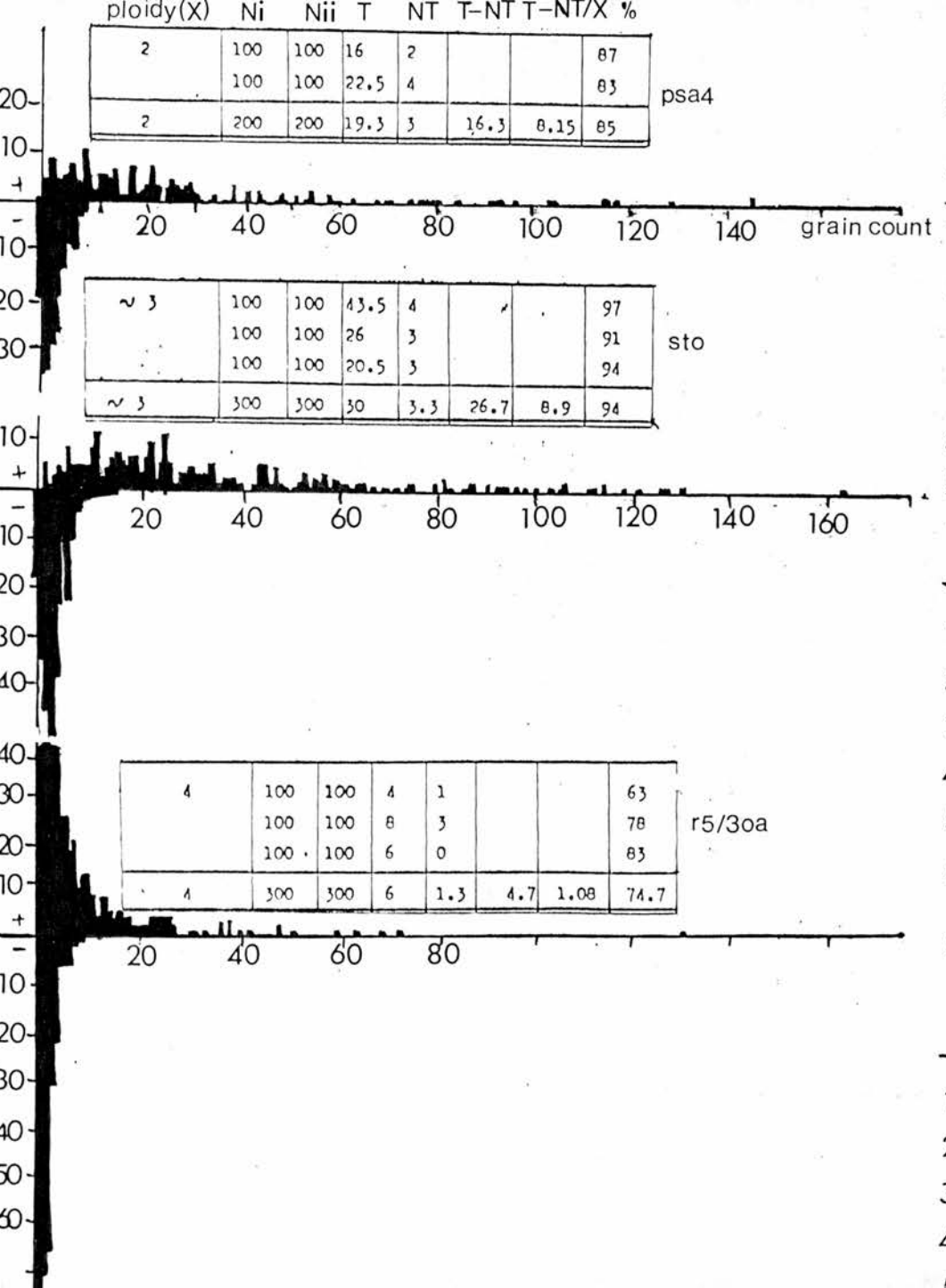
MEASUREMENT OF HOMOTYPIC METABOLIC COOPERATION CAPACITIES OF HYBRID AND CONTROL CELL LINES USING A TRITIATED URIDINE TRANSFER ASSAY

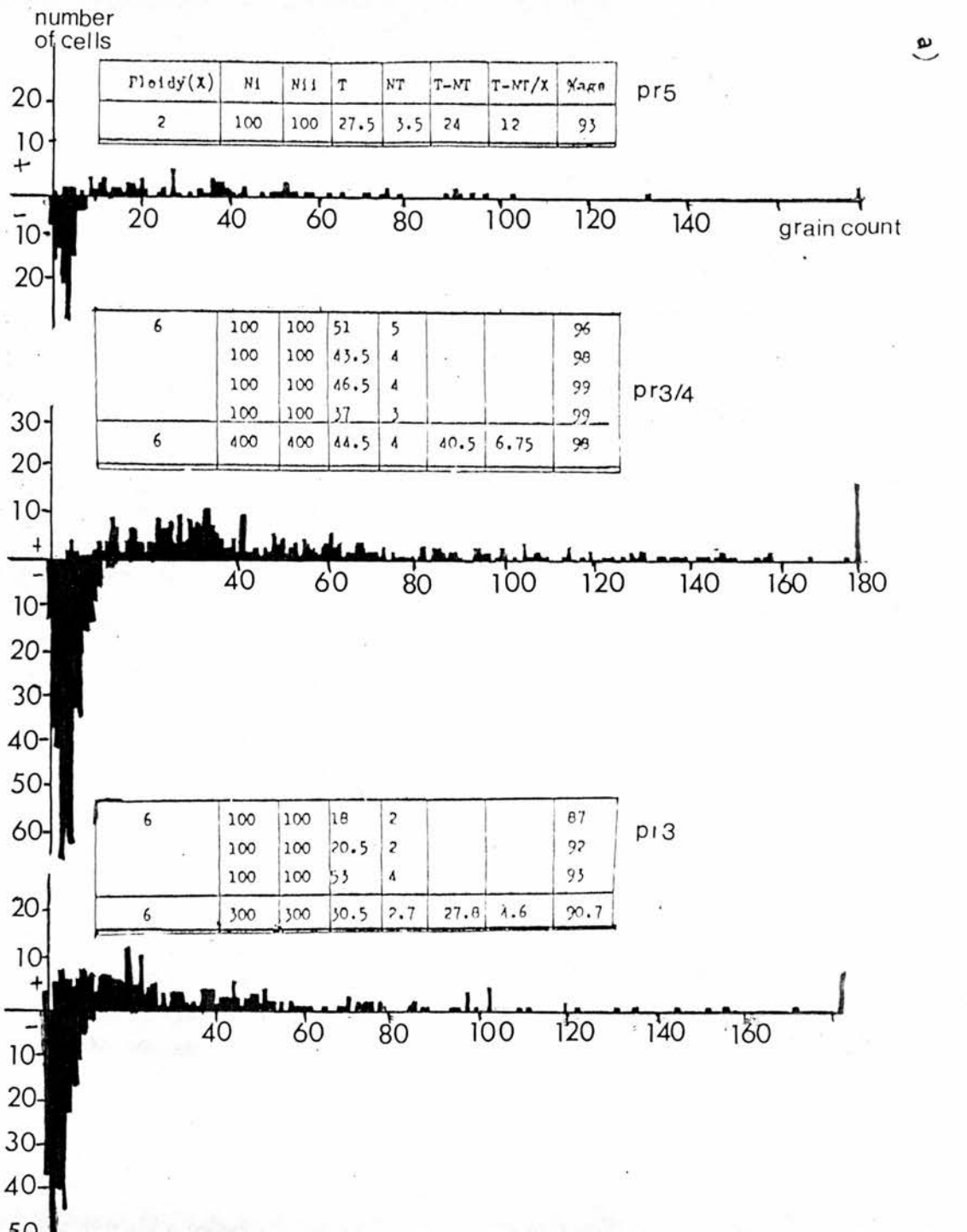
Shows the histogram representation of four separate autoradiograph experiments in which the homotypic transfer of tritiated uridine was measured in the cell lines PR3,PR3/4,PR5,PSA4,R5/3OA and (in one experiment) STO.Histogram plots show the result of counting the number of grains in cells which were in contact (touching cells) with donor cells (identified as heavily labelled cells) compared to the number of grains in an isolated recipient cell in the same microscpe field which was not in contact (NT) with a donor cell.

The resulting pairs of cells were then plotted onto the histograms with the touching cells (T) above the horizontal axis and the control (NT) background cells below this axis.Histogram plots represent the collated results of counting from 2-4 dishes per cell line in each experiment and each line in the tabulated inset represents one dish of that experiment.Each of the four sets of histograms 8a,8b,8c, and 8d is a separate experiment. Key:

```
Ploidy (X) - ploidy of the cell line
        Ni - Number of test cells counted
       Nii - Number of control cells counted
         T - Median number of grains in test cells
        NT - Median number of grains in control cells
      T-NT - Test cell grain count adjusted for background
    T-NT/X - Test cell grain count adjusted for both
             background and for ploidy
     %age - Percentage of test cells cooperating
             (computor analysis)
```

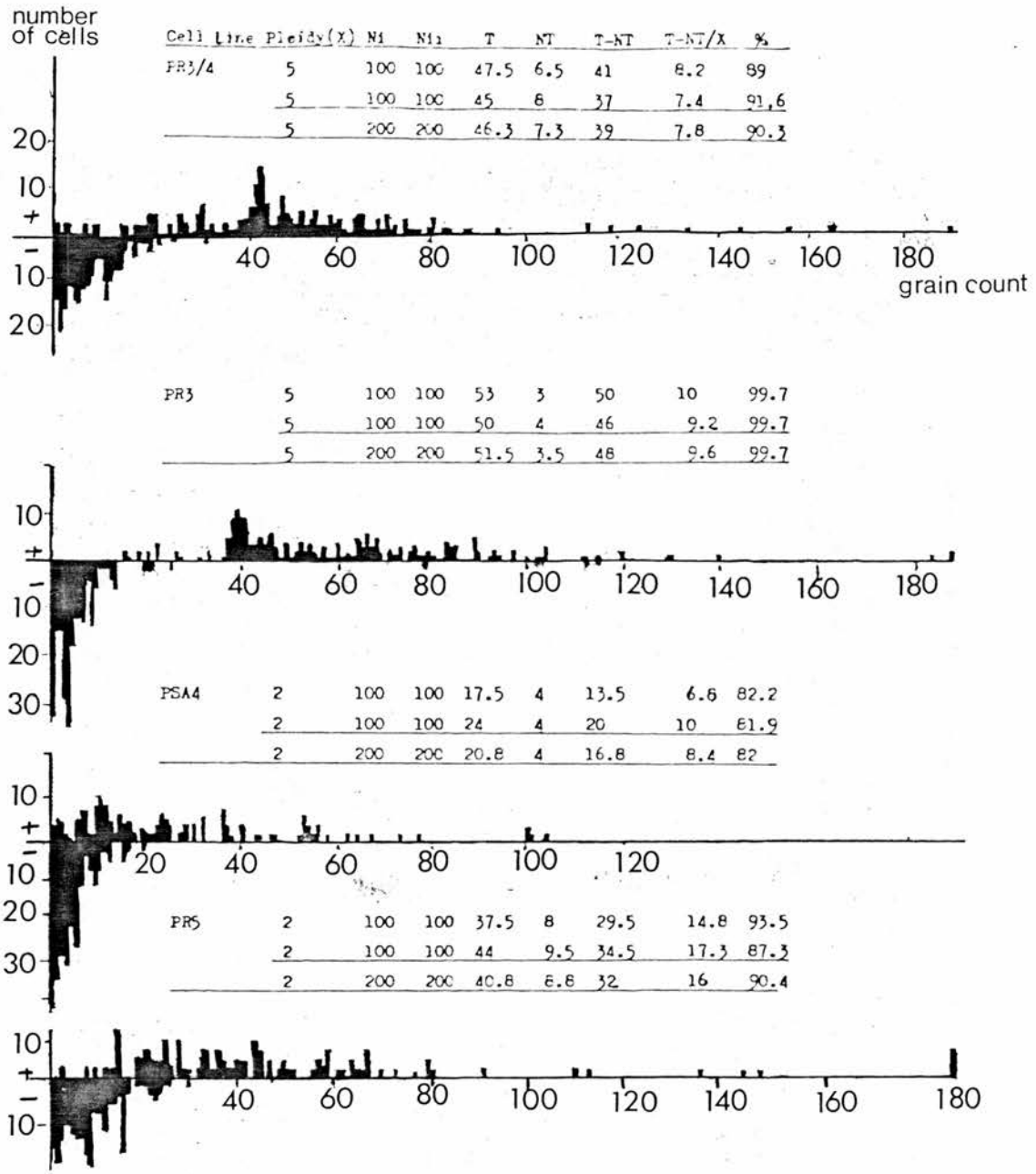
Note: The experiments 8c and 8d correspond respectively to the experiments 27/3 and 9/4 which can be directly compared to the experiments in chapter 4 (11a and 11b) and in chapter 5 (4c and 4d) which were set up at the same times as these two experiments.

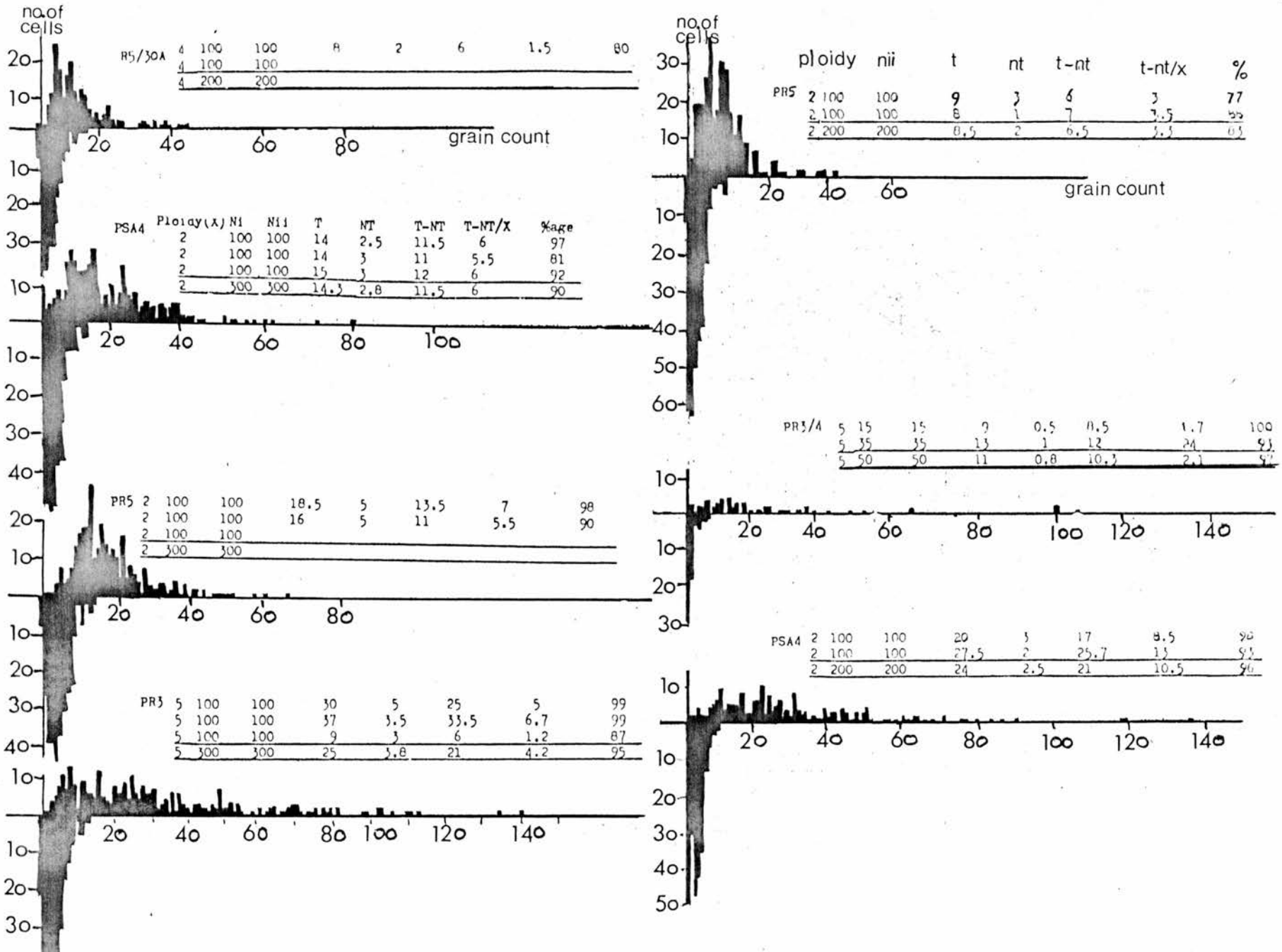

ploidy(X) Ni Nii T NT T-NT T-NT/X %
2 100 100 16 2 87
100 100 22.5 4 83
2 200 200 19.3 3 16.3 8.15 85
psa4
~ 3 100 100 43.5 4 97
100 100 26 3 91
100 100 20.5 3 94
~ 3 300 300 30 3.3 26.7 8.9 94
sto
4 100 100 4 1 63
100 100 8 3 78
100 100 6 0 83
4 300 300 6 1.3 4.7 1.08 74.7
r5/3oa
grain count


number of cells
Ploidy(X) Ni Nii T NT T-NT T-NT/X %age
2 100 100 27.5 3.5 24 12 93
pr5
6 100 100 51 5 96
100 100 43.5 4 98
100 100 46.5 4 99
100 100 37 3 99
6 400 400 44.5 4 40.5 6.75 98
pr3/4
6 100 100 18 2 87
100 100 20.5 2 92
100 100 33 4 93
6 300 300 30.5 2.7 27.8 4.6 90.7
pr3
grain count

a)

b)


number of cells
grain count
Cell Line Ploidy(X) N1 N12 T NT T-NT T-NT/X %
PR3/4 5 100 100 47.5 6.5 41 8.2 89
5 100 100 45 8 37 7.4 91,6
5 200 200 46.3 7.3 39 7.8 90.3
PR3 5 100 100 53 3 50 10 99.7
5 100 100 50 4 46 9.2 99.7
5 200 200 51.5 3.5 48 9.6 99.7
PSA4 2 100 100 17.5 4 13.5 6.6 82.2
2 100 100 24 4 20 10 81.9
2 200 200 20.8 4 16.8 8.4 82
PR5 2 100 100 37.5 8 29.5 14.8 93.5
2 100 100 44 9.5 34.5 17.3 87.3
2 200 200 40.8 8.8 32 16 90.4


(a)
no.of cells
grain count
R5/30A
PSA4
Ploidy(x) Ni Nii T NT T-NT T-NT/X %age
2 100 100 14 2.5 11.5 6 97
2 100 100 14 3 11 5.5 81
2 100 100 15 3 12 6 92
2 300 300 14.3 2.8 11.5 6 90
PR5
PR3
(c)
no.of cells
grain count
ploidy nii t nt t-nt t-nt/x %
PR5
PR3/4
PSA4

The two hybrid lines PR3 and PR3/4 are both consistently shown (4 experiments) to have a high level of homotypic cooperation.For the line PR3 the range of percentage cooperation estimates varies between 87 and 99% while T-NT/X values vary from 1.2-6.7.Metabolic cooperation of the hybrid and their parental lines indicate that the hybrid lines can cooperate homotypically and therefore their gap junctions are capable of mediating the transfer of uridine nucleotides,between cells of the same cell line.

Results of tritiated uridine autoradiography experiments show that both of the hybrid lines PR3 and PR3/4,are capable of metabolic cooperation.It seems clear however that the amount of cooperation is slightly reduced as compared to both PSA4 (the mec+ parent line).These results,together with those of chapters 4 and 5 are collated in chapter 5.5. and are further discussed (in context with results of Chapters 4 and 5) in Chapter 6.

3.5. Differentiation of Hybrid Lines in Comparison to Parent Lines

The differentiation properties of hybrid lines derived from the ec cell lines R5/3OA and PSA4 are of interest both to discover whether or not the lack of differentiation found in R5/3OA cells would be transmitted to hybrid progeny,i.e. whether this is a dominant trait,and also as a means of investigating the relationship between Metabolic Cooperation and differentiation.The differentiation properties of the hybrid lines PR3 and PR3/4 have been investigated in three different ways.

The first,which is further discussed in Chapter 5,involves the assessment of colonies plated onto gelatin in STO feeder conditioned medium.Section 3.5.1 reports results of observation of colony morphology of cells plated in STO feeder conditioned medium. *In vitro* differentiation was also investigated [3.5.2] by the analysis of embryoid bodies [see 1.5] for endoderm differentiation and cavity formation. *in vivo* differentiation was investigated by the analysis of tissues found in teratocarcinomas [1.4] formed by the injection of cells of these lines into syngenic mice.

3.5.1. Colony Morphology

It was observed,when cells from the hybrid lines PR3 or PR3/4 were plated onto gelatin and grown in medium conditioned with STO feeders [2.3.3] for 4-5 days that the colonies forming had a very variable appearance within one dish.Some colonies appeared to be composed entirely of ec cells while others appeared to be completely differentiated and the remainder formed an intermediate category of colonies which were partially differentiated (see Chapter 5,Figure 5:1 for illustrations).

This observation is in contrast to the behaviour of parental lines under such conditions,R5/3OA cells form colonies of which all or almost all are tightly packed and appear to consist of ec cells alone.PSA4 cells form colonies showing a wider range of variation than that displayed by R5/3OA colonies,but the majority fall into the intermediate category showing partial differentiation of colonies with a central core of ec cells.This therefore does not correspond with the extensive heterogeneity found with the hybrid lines.

3.5.2. Embryoid Body Differentiation

Embryoid bodies were made from cells of the hybrid lines PR3 and PR3/4 and also from PR5 and from the parental lines R5/3OA and PSA4 using the normal procedure as described in Chapter 2 [2.2.10].H+E stained paraffin sections of these preparations were then examined under the microscope and scored for the extent of their differentiation.This process is described in detail in the methods Chapter [2.4.5].Results of these counts are placed in Figure 3:9 together with pie chart illustration.

Figures 3:10 illustrate the H+E stained sections of EBs as seen at low power under the microscope (x10) and also representative individual pictures of these EBs at the higher power (x40) at which the sections were scored.

A category of embryoid bodies not found in EBs from the lines with greater potency was identified in the line R5/3OA which consisted of embryoid bodies with considerable eosin staining,some of which was due to peripheral necrosis and some of which was associate with rounded cells around the margin of the EB which could not be positively identified as endoderm formation.As Table 3:3

Figure 3:9

EMBRYOID BODY DIFFERENTIATION

a) Results of embryoid body counts showing numbers of EB's in each classification.

b) Pie charts illustrating the extent of differentiation occurring in R5 3OA,PSA4,PR5,PR3 and PR3 4.

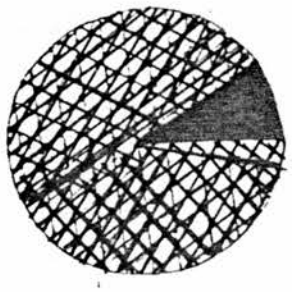
Key:

1.1 Undifferentiated
1.11 Undifferentiated but associated with eosin staining and necrotic cells
2. VE and PE +
3. VE and PE ++
4. VE and PE and Cavity +
5. VE and PE and Cavity ++

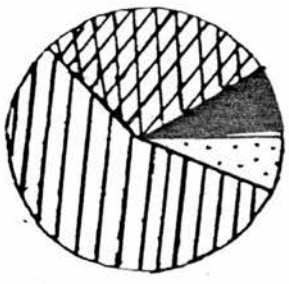
See Chapter 2,Figure 2:1 for definition of the five classes of extent of differentiation

a)

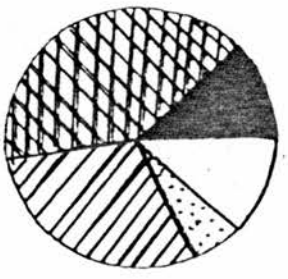
Cell line	1 Undifferentiated 1	11	2 VE + PE	3 VE ++ PE	4 VE PE cavity +	5 VE PE cavity ++
PR3	11	-	33	25	2	9
	12	-	37	27	8	12
	23	-	70	52	10	21
PR5	5	-	21	54	27	2
PR3/4	10	-	63	81	17	-
	25	-	58	108	8	-
	11	-	51	99	14	1
	14	-	39	126	10	-
	60	-	211	414	49	1
PSA4	-	-	3	76	41	6
	2	-	15	102	44	11
	2	-	18	178	85	17
R5/30A	29	201	5	-	-	-
	29	238	4	-	-	-
	54	445	9	-	-	-



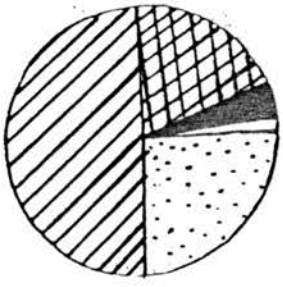
R5/30A



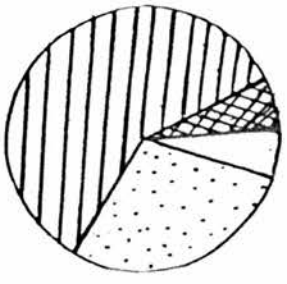
PR3/4



PR3



PR5



PSA4

Figure 3:10

EMBRYOID BODY DIFFERENTIATION IN THE CELL LINES R5/3OA AND PSA4

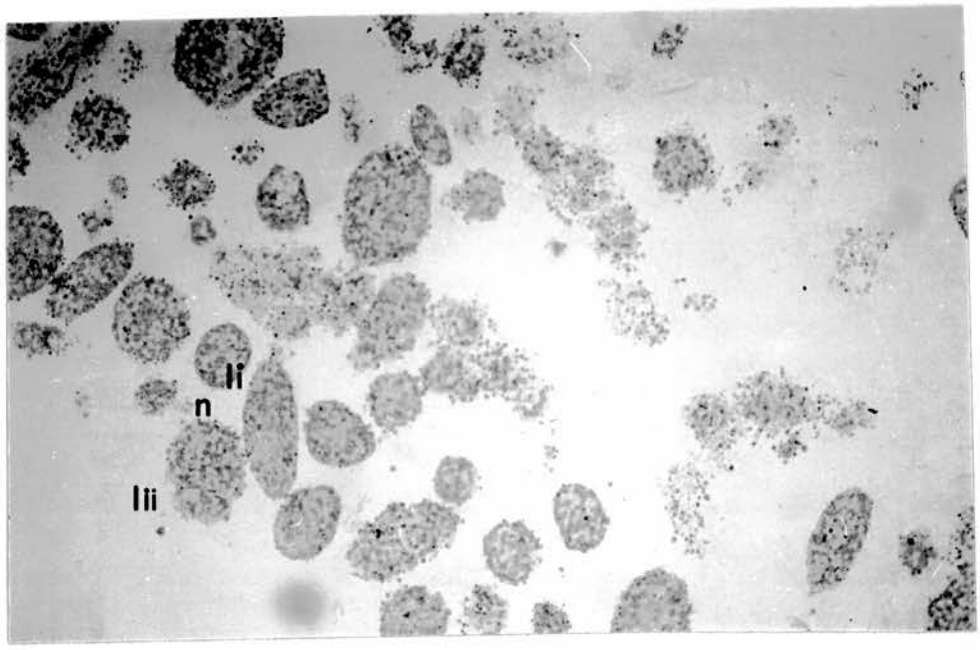
a) Embryoid bodies from aggregates of cells from R5/3OA illustrating the undifferentiated categories (1i) and (1ii). print magnification was 4x - Overall magnification = 800

b) Embryoid bodies from aggregates of cells from PR3 illustrating all four categories of differentiation (2-5) and the undifferentiated category (1i). print magnification was 4x - Overall magnification = 800

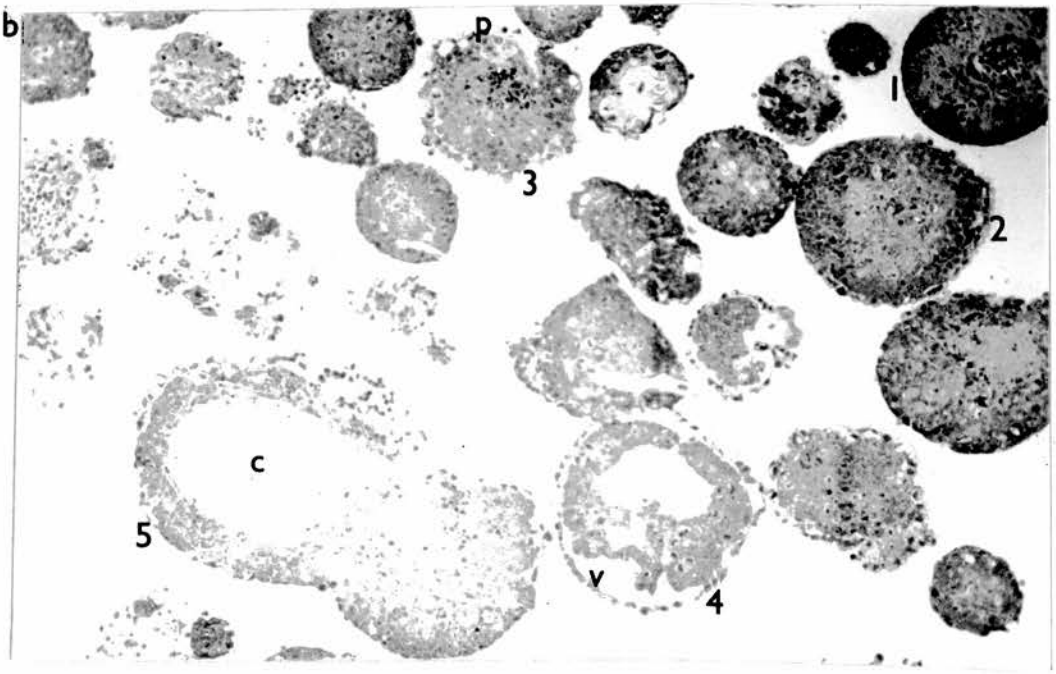
Key:

ec - embryonal carcinoma cells
c - cavity
p - parietal endoderm
v - visceral endoderm
n - necrosis

a



b



illustrates,all of the lines PR3,PR3/4 and PR5 are capable of a greater extent of differentiation in EB suspensions than is the parental line R5/3OA.In comparison with the other parental line (PSA4) however,which is pluripotent,the extent of differentiation (as measured by the extent of endoderm differentiation and proportion of cavitating EBs) is lower in all three PR lines.

The line PR3/4 shows the least differentiation in this respect,although the proportion of PR3/4 EBs in the 1st and 2nd categories is lower than is found in PR3,this is because more than 50% of PR3/4 EBs fall into the 3rd category having extensive VE and PE differentiation but no cavitation.

3.5.3. Tumour Formation

Teratocarcinoma-type tumours were formed [Table 3:3] at the site of injection (left shoulder) in syngenic mice injected with cells of the lines PR5,PR3 and PSA4.No tumours were formed in any of the three mice injected with the cell line R5/3OA,however,previously [M.L.Hooper pers.comm.] tumours in mice have been obtained from this line and sections of five tumours obtained in this way [M.L.Hooper] were also scored as a control for the hybrid lines [Table 3:4].

H&E stained slides of sectioned tumours were scored for the presence or absence of various differentiated cell types [Table 3:4] and also for the presence or absence of ec cells.The results are consistent with those obtained from *in vitro* differentiation experiments [3.5.1,3.5.2].Tumours derived from the cell lines PR3,PR5 and PSA4 were all found to be well differentiated and displayed differentiated tissue derived from all three germ cell layers although all three cell types seemed to produce tumours containing large areas of muscle tissue.Cartilage,keratinising epithelium (skin) and smooth and striated muscle were found in all tumours examined derived from either PR3,PR5 or PSA4 cells.In addition to these tissue types PSA4 derived tumours commonly contained tissue of bone,glandular epithelium and ciliated epithelium (respiratory) and some also had haematopoesis,putative trophectoderm,neural tube formation and unidentified columnar epithelium.

In addition to these differentiated cell types tumours derived from PR3,PR5 and PSA4 all had a small residual core of undifferentiated ec cells.Tumours derived from the parent line R5/3OA were,in contrast composed predominantly of undifferentiated ec cells.All sections of tumours from this line were also found

Table 3:3

A SUMMARY OF TUMOUR FORMATION IN SYNGENIC MICE INJECTED WITH CELLS OF THE LINES PSA4,R5/3OA,PR3 AND PR5

Shows the fate of 16 mice injected with cells of one of the lines PR3,PR5,PSA4 and R5/3OA. Key:

F - female mouse
M - male mouse
N A - not applicable
n.o.a.b. - no other abnormality found after autopsy

Tumours discovered were all teratocarcinomas and were all sited on the left shoulder of the animals (where the cells were originally injected).Several of the tumour bearing mice were found to have enlarged and inflated uterine horns, this was identified as cystic displasia and is indicated in the remarks where appropriate.

Tumours Formed per Mouse Injected

R5 3OA : 0/3
PSA4 : 4/4
PR3 : 2/4
PR5 : 5/5

note: Blood supply was identified by evidence of vasculation as well as the presence of large quantities of blood.

Cell line	passage number	sex of animal	days to form	remarks
R5/30A	16	F	N/A	no tumour after 12 months
	16	M	N/A	no tumour after 12 months
	16	M	N/A	no tumour after 12 months
PSA4	14	F	25	2cm tumour with a large blood supply cystic dysplasia of the uterus
	14	M	34	2.5cm tumour with a large blood supply n.o.a.b.
	14	F	49	3cm tumour with good blood supply;cystic dysplasia of the uterus
	14	M	74	1.5cm tumour;n.o.a.b.
PR5	7	F	25	2cm tumour with a large blood supply cystic dysplasia of the uterus
	7	F	25	2cm tumour;cystic dysplasia of uterus
	7	M	34	1-2cm tumour with a large blood supply n.o.a.b.
	7	M	42	1.5cm tumour;n.o.a.b.
	7	M	85	2cm tumour;n.o.a.b.
PR3	5	F	34	1.5cm tumour;n.o.a.b.
	5	M	74	2.5cm tumour;n.o.a.b.
	5	F	N/A	no tumour after 12 months
	5	M	N/A	no tumour after 12 months

Table 3:4

ANALYSIS OF THE DIFFERENTIATION FOUND IN MOUSE TERATOCARCINOMAS DERIVED FROM PSA4,R5/3OA AND THEIR DERIVATIVE LINES PR3 AND PR5

Shows the results of scoring mouse tumours for the presence or absence of eleven categories of differentiated tissue and also for the presence or absence of undifferentiated ec cells.

Tumours derived from the lines PR3,PR5 and PSA4 are well differ- entiated with most categories of tissue type present in all tumours. Tumours from the line R5/3OA however are poorly differentiated

Key:

1 - cartilage
2 - bone
3 - haematopoeisis
4 - putative trophectoderm
5 - neural tube formation
6 - glandular epithelium
7 - keratinising epithelium
8 - ciliated epithelium
9 - smooth muscle
10 - striated muscle
11 - columnar epithelium
(12 - embryonal carcinoma cells)

notes:

i) plus signs in brackets -(+)- indicate that this category is present in some but not all tumours scored

ii) double plus signs - ++ - indicate that the cell type (ec) is the predominant tissue in the tumour

iii) plus signs - + - indicate presence and minus signs *-* indicate absence of a tissue type.

Cell Line	1	2	3	4	5	6	7	8	9	10	11	(12)
PSA4	+	+	−	−	−	+	+	+	+	+	+	+
	+	+	+	−	−	+	+	+	+	+	+	+
	+	+	+	−	+	+	+	+	+	+	−	+
	+	−	−	−	+	+	+	+	+	+	−	+
	+	(+)	(+)	−	(+)	+	+	+	+	+	(+)	+
R5/30A	−	−	−	−	−	−	−	−	−	+	−	++
	−	−	−	−	−	−	−	−	−	+	−	++
	+	−	−	−	−	−	−	−	+	+	−	++
	−	−	−	−	−	−	−	−	+	+	−	++
	−	−	−	−	−	−	−	−	−	+	−	++
	(+)	−	−	−	−	−	(+)	−	(+)	+	−	++
PR3	+	−	−	−	−	−	−	+	+	+	−	−
	+	+	+	−	−	+	+	+	+	+	+	+
	+	(+)	(+)	−	−	(+)	(+)	+	+	+	(+)	(+)
PR5	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	−	−	−	+	+	−	+	+	−	+
	+	+	−	−	+	+	+	+	+	+	−	+
	+	+	+	−	−	+	+	+	+	+	−	+
	+	+	−	−	−	+	+	+	+	+	−	+
	+	+	(+)	(+)	(+)	+	+	(+)	+	+	(+)	+

to contain striated muscle and some sections were also found to contain small areas of cartilage,smooth muscle.

Tumours derived from the fusogen treated diploid line PR5 appeared to be slightly more differentiated than those of PSA4 cells although they showed the same general pattern of differentiation.PR5 tumours commonly contained cartilage,bone,glandular epithelium,ciliated epithelium,columnar epithelium and smooth and striated muscle.In addition some tumours also had haematopoiesis associated with the bone production.Neural tube formation and putative trophectoderm were also found.

Tumours derived from one tumour,of PR5 cells,in particular showed extensive differentiation into bone with large areas of productive bone marrow with evidence of granulocytes (lymphopoiesis) myelocytes (myelopoiesis) and with large lakes of erythrocytes containing nucleated precusor cells indicating erythropoiesis an additional slide of this section was stained with giemsa to show up these features more clearly and is illustrated in Figure 3:11.This figure also illustrates typical examples of other differentiated tissues found in these tumours.

Tumours derived from the hybrid line PR3 were possibly not as extensively differentiated as those from the lines PR5 and PSA4 but this was not quantified and so was difficult to assess.Tumours of this line commonly had muscle (smooth and striated),cartilage,and keratinising epithelium.Some of these tumours also contained bone,neural tube formation,glandular epithelium,ciliated epithelium and columnar epithelium.

Several of the tumour bearing mice reported in this section [Table 3:3] were found on autopsy to have distended and inflamed uterine horns.This disorder was identified as cystic dysplasia and the paraffin section [Figure 3:12] of one of these uteri shows the highly convoluted glandular composition of the uterine walls which may possibly have had some effect on the production of tumours in these animals.

3.6. Summary

Methods for the isolation of hybrid lines from PEG fusion of cells were modified so that cell lines could be isolated from the fusion of two ec

Figure 3:11

DIFFERENTIATION OF MOUSE TERATOCARCINOMAS FROM THE LINES PSA4,R5/30A AND THEIR DERIVATIVE LINES PR5 AND PR3

Illustrates examples of differentiated tissues found in tumours of the cell lines PSA4,PR3 and PR5.Unless indicated sections were stained with H&E.

a) Keratinising epithelium:an outer layer of epithelial cells (arrow) encloses cells which have become completely keratinised and have lost their nuclei,this corresponds to skin formation.(magnification = 800)

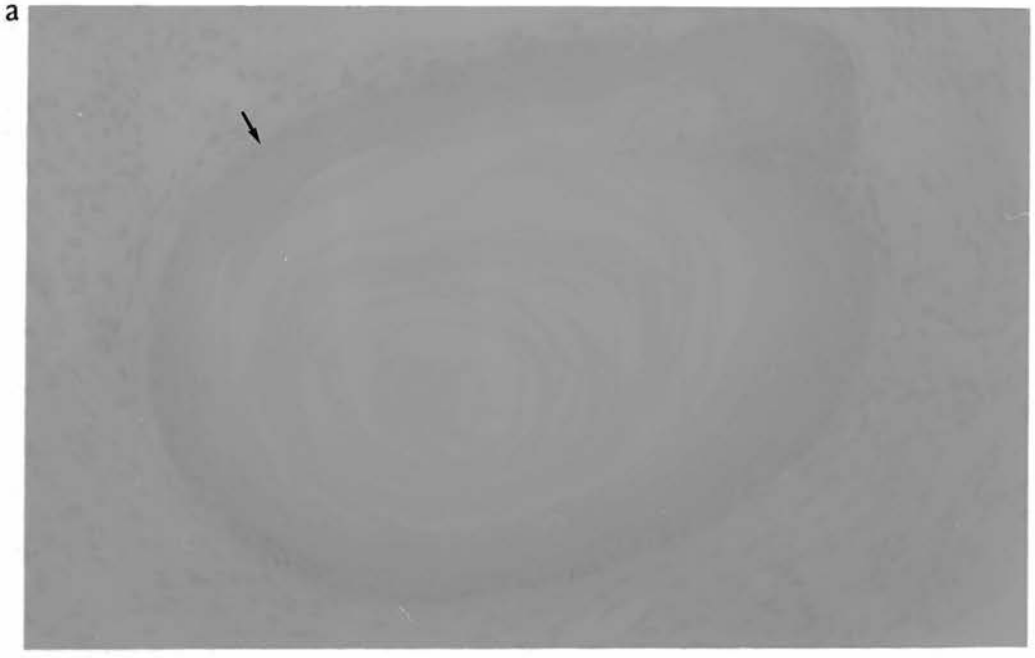
b) Mixed glandular and ciliated epithelium : High magnification of epithelium which is largely columnar and consisting of a majority of cells which are ciliated on their inner edge (c) with some cells which are swollen and appear white in H&E stained slides,these are secreting epithelial cells (g).(magnification = 3200)

c) Neural tube formation characterised by the accentric position of the nuclei.Mitotic figures are indicated by arrows.(magnification =1600)

d) Illustrates striated muscle (M) and adipose tissue (**a**).(magnif- ication = 1600)

e) low power view of haematopoiesis and bone formation (Giemsa stains) showing lakes of erythrocytes (**e**),dark staining cells are erythrocyte precursor cells indicating productive bone marrow function (small arrow).Large arrow indicates bone.(magnification = 800)

f) High power magnification (3200) of haematopoeisis,arrows indicate cells with mulilobed nuclei which are precursor cells of lymphocytes.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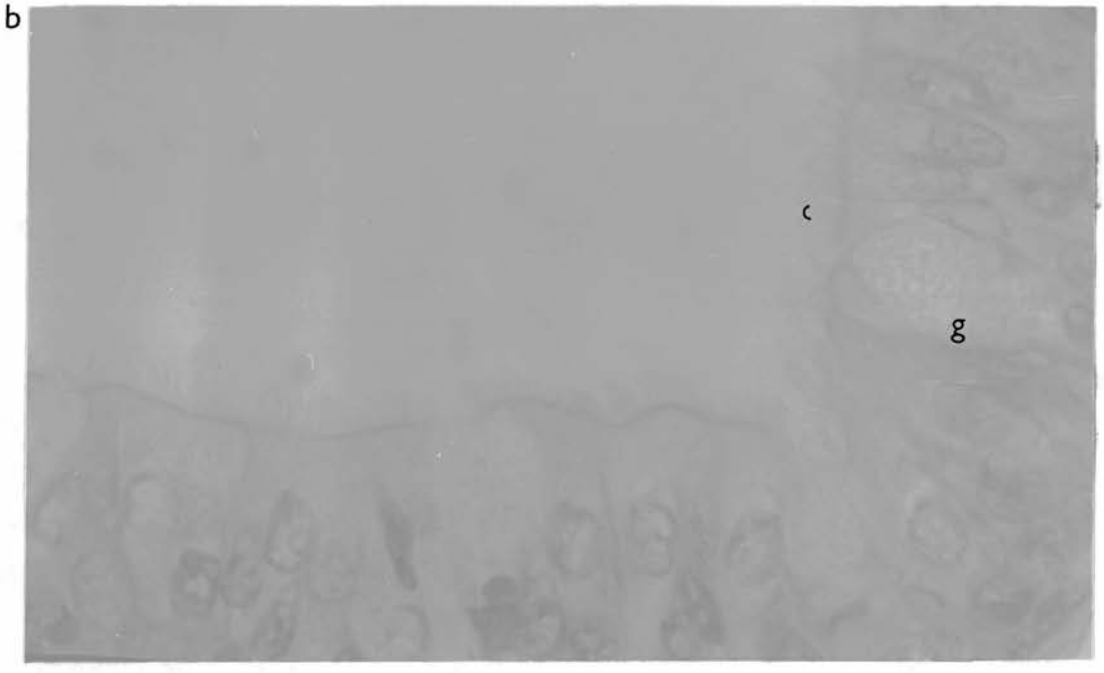
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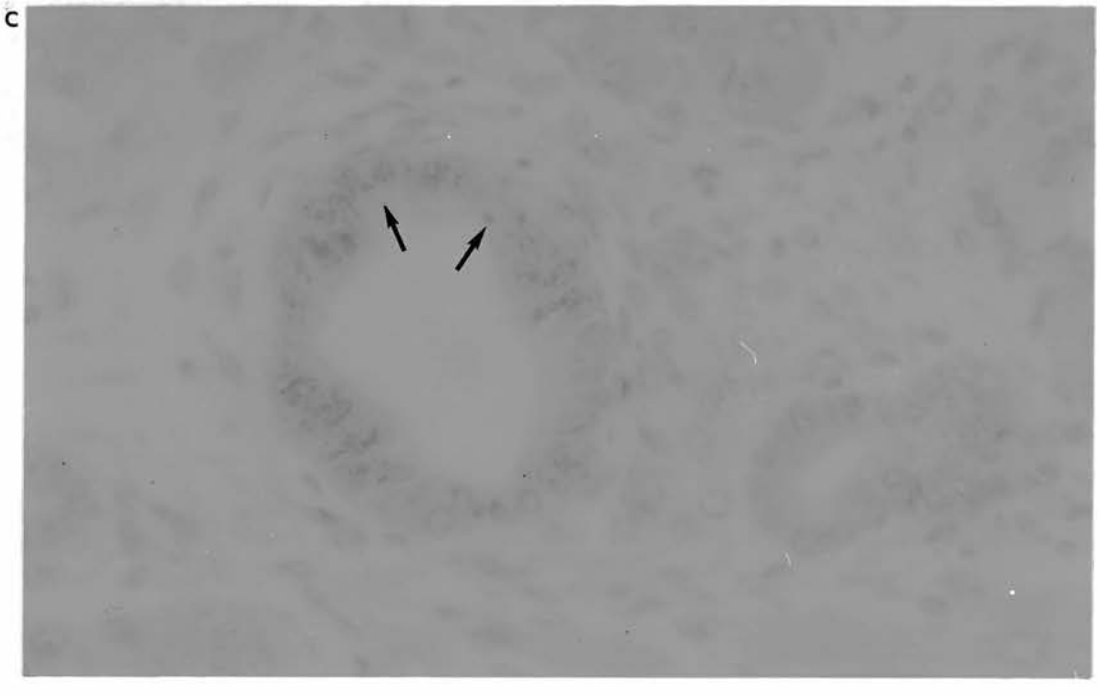
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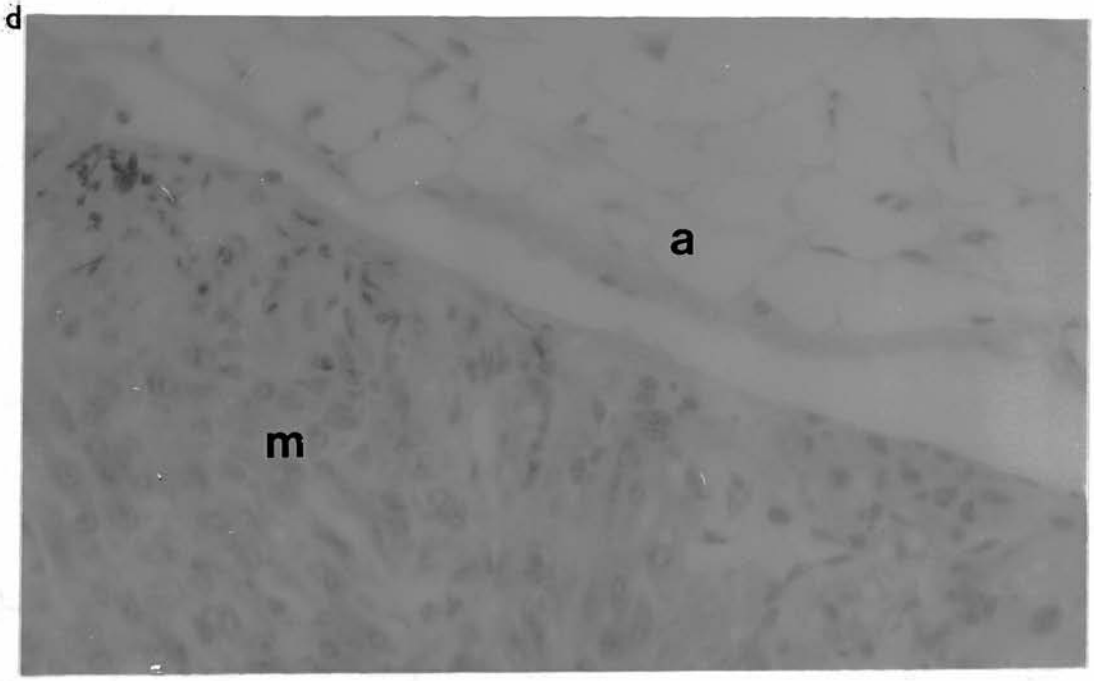

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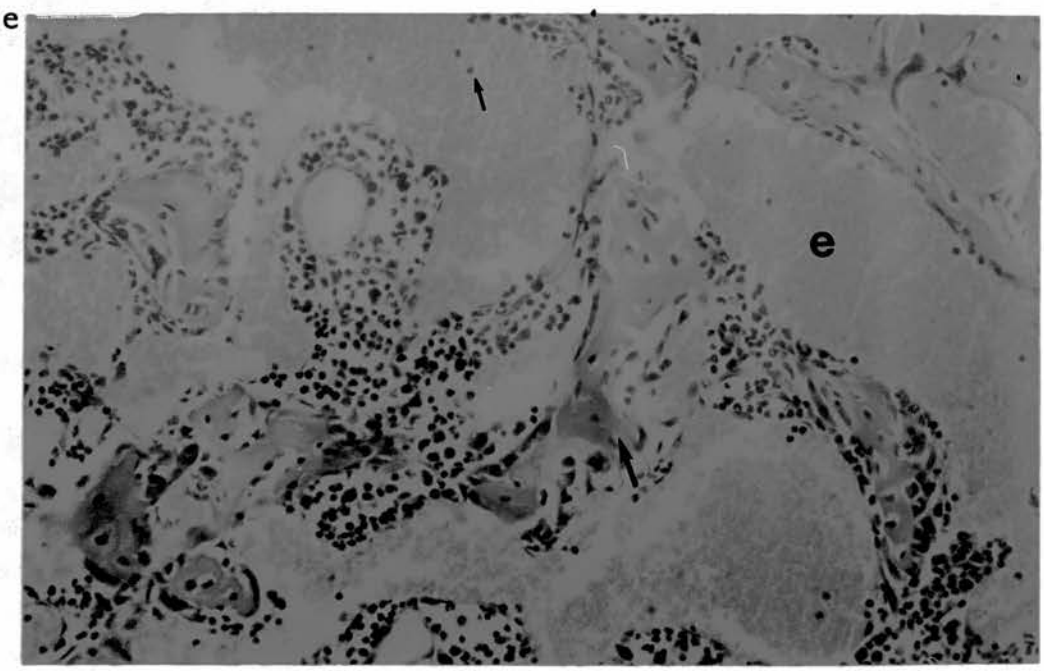

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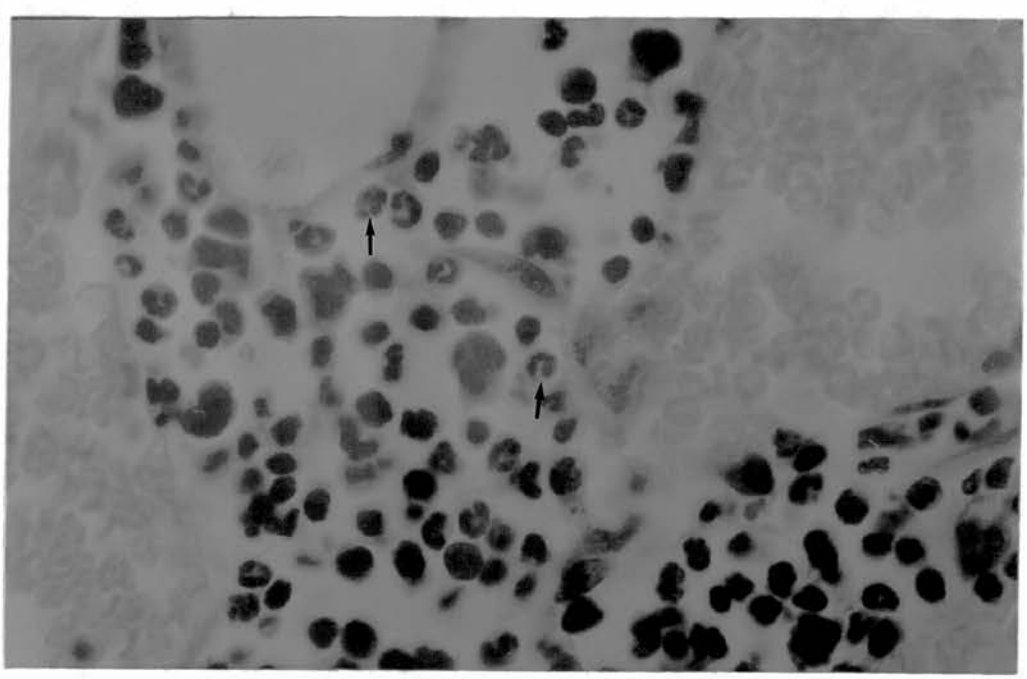
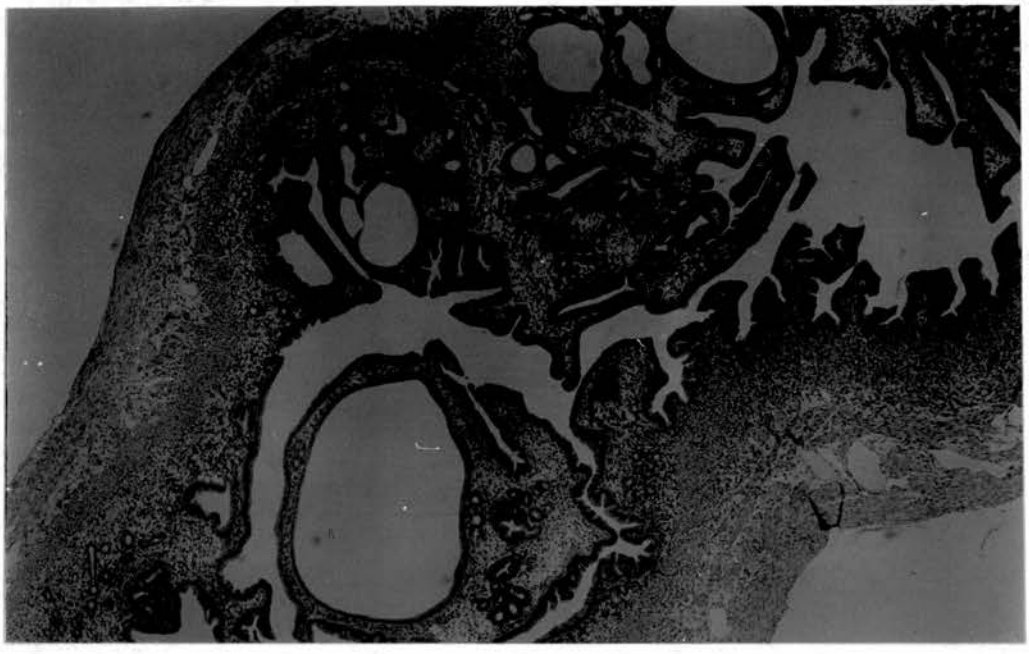


Figure 3:12

ILLUSTRATION OF UTERINE CYSTIC DYSPLASIA IN A TERATOCARCINOMA BEARING MOUSE

Shows the extensive distension of the uterine walls and the increase in glandular secretion found in several mice bearing teratocarcinomas derived from the cell lines PSA4 or PR5.

(Magnification = 128)



cells.One of the cell lines involved,PSA4,was feeder dependent.Early fusion experiments using STO feeders [101] proved that these Ouabain sensitive PSA4 cells were being selectively "rescued" from Ouabain toxicity when grown in HAT + Oua medium, by metabolic cooperation with the Oua Resistant STO feeder cells.The R5/3OA cells were Mec- and therefore could not be rescued from the HAT medium in this way.

Therefore,because of this feeder rescue,the use of STO cell conditioned medium [154,155] was introduced and modified.After several plating efficiency experiments involving both feeder conditioned and "live" STO conditioned media it was concluded that the easiest,most reproducible and effective method of STO cell conditioning was to incubate freshly mitomycin-C treated STO feeder cells with CM(10) for 7 days.The efficiency of this medium was then further improved by adding Gln,B-mercaptoethanol (10^{-4} M) and FCS (5- 10%) after the filtering of the medium.

The use of STO feeder conditioned medium prevented selective feeder rescue but,despite the observation of bi-nucleate cells in coverslip preparations of PEG 6000 treated cells and also for the first 2-3 days in the dishes plated out from fusion experiments,there was no survival of such cells after the first few days.

The two most obvious reasons for failure of binucleate cells to survive were; i) That cells may have been only fusing homotypically with cells of their own line,or that for some reason hybrids of these two cell types were incompatible with survival. ii) That culture conditions were inadequate,or fusion treatment too harsh,to allow for the survival of heterokaryons through the first division and that for this reason no hybrid cells could grow.

There is no definite way of excluding the first possibility except by the production of hybrid lines and although it seems unlikely that cells were only fusing homotypically as there are no known differences between the membranes of R5/3OA and PSA4 which could prevent their fusion with each other,it is quite plausible that even cells of the same type (ec) are not compatible in hybrid form.The inclusion of DMSO (10%) into the fusion treatment [121] and reduction of the time of exposure to the fusogen had no effect on bi-nucleate cell survival,which indicated that it was at the heterokaryon survival stage rather than at formation where the block was occurring.

The second possibility for failure of binucleate cell survival seems more plausible and an experiment was devised to overcome most of these difficulties.Therefore the culture conditions were improved by using "enriched conditioned medium" which contained 20% FCS and 10^{-4} M B-mercaptoethanol,a method already shown to improve the survival chances of cells isolated from mouse blastocysts [17].

The post-fusion procedure was altered so that fusogen treated cells were not trypsinised until 7 days after treatment,therefore giving possible heterokaryons a chance to recover from fusogen treatment before undergoing the trauma of trypsinisation.This procedure unfortunately means that cell lines derived in this way will not be clonal and so may be derived from more than one fusion event.This disadvantage could be overcome by cloning of the lines.The isolation of variants by picking growing colonies of cells effectively clones them and so all of the lines used in this study (with the exception of PR3,PR3/4 and PR5) are clonal lines.

The fusogen used in this set of experiments (50% PEG 1000 in HEPES buffer) was reported to be the most effective of a series of fusogen treatments tested by Klebe and Mancuso [78] which included DMSO and PEG 6000.Coverslip counts of PSA4 and R5/3OA cells fused with PEG 1000 in HEPES buffer showed approximately twice as many bi-nucleate cells (14%) with this treatment as were found in early coverslip counts using cells treated with PEG 6000 (7%).These results therefore support the conclusions made by Klebe and Mancuso.

The first putative hybrid cell line to be isolated (PR1) was unfortunately lost through bacterial contamination before a permanent stock was established, but it was karyotyped.Chromosome counts of this line indicated that it was approximately hexaploid with 2 metacentric chromosomes and therefore seems likely to have been an R5/3OA + PSA4 cell Hybrid.

Repeat experiments using this procedure resulted in the isolation of four more cell lines,one was lost through contamination (PR2),two (PR3 and PR3/4) were shown to be sub-hexaploid with 2 metacentric marker chromosomes and therefore putative hybrid lines and one (PR5) was shown to be a diploid Ouabain resistant derivative of PSA4.This last cell line grew poorly and was routinely maintained in 20% FCS,it was characterised in parallel with the

putative hybrid lines and the parent lines as it serves as an internal control for PEG 1000 fusogen treatment.

All of the lines (PR3,PR3/4 and PR5) isolated from PSA4/R5:3OA fusogen co-culture experiments were shown to be resistant to both HAT and Ouabain although there was considerable variablility in the plating efficiencies of these experiments.This is thought to be because the $LD_{50}$for these cells in ouabain is around 3mM (at which concentration all of these experiments were formed) so that resistance was measured on a steeply sloping part of the survival curve resulting in large variation in cell survival with small changes in resistance due to physiological variation such as,for example,would occur with small fluctuations in pH and changes in the quality of conditioned medium (this latter would not apply to R5/3OA).This could be investigated (in the case of ouabain) by examining the resistance of these lines in lower concentrations of ouabain.

The two lines PR3 and PR3/4 were shown to have modal chromosome numbers of 102 and 100 respectively and to both have the metacentric marker chromosomes from the R5/3OA parent.Their morphology was ec cell type and consistent with being intermediate between that of R5/3OA and PSA4,although like the latter they were both STO feeder cell dependent.They were both routinely maintained on STO feeder layers in 10% FCS supplemented with B-mercaptoethanol (10^{-4} M) and grew well under these conditions.

Both PR3 and PR3/4 were shown to differentiate spontaneously,in STO conditioned medium,in suspended embryoid bodies and,*in vivo*,in differentiated teratocarcinomas.Analysis of sectioned EBs indicated that this differentiative ability was intermediate between PSA4 and R5/3OA,although whether this was due to a combination of the R5/3OA and PSA4 phenotypes or due to other factors,such as ploidy or fusogen treatment was not conclusively determined.The fusogen treated,PSA4 derived line PR5 also showed a slight decrease in differentiative ability in EB suspensions although this was not as marked as that found in PR3 and PR3/4.

In vivo differentiation experiments (tumour formation) confirm the dominance of the differentiating (PSA4) phenotype.A slight reduction was observed in the extent of differentiation of the hybrid line PR3 when compared to the differentiating parent line PSA4,while the non-hybrid (fusogen treated) line PR5 showed a slight increase in differentiated cell types.

However,these observations were not quantified because of the impossibility of identifying all of the tissue types present,so that any conclusion about the comparative extent of differentiation in well differentiated tissues must be treated with caution.

However the phenotype of the well differentiated tumour can be clearly distinguished from tumours derived from lines such as R5/3OA which are predominantly formed of ec type cells and are very poorly differentiated.

Results of Uridine transfer analysis of metabolic cooperation indicate that both PR3 and PR3/4 are capable of gap junction mediated communication but that,when ploidy is taken into account,this function is somewhat reduced when compared to the PSA4 (mec+) line.The period of donor and recipient incubation may be crucial in this analysis as the line PR5 shows a marked decrease in cooperation function in experiments where the donor an recipient cells were incubated for 3 hours only as compared to 4 hour incubation.

All of these results are consistent with the two lines PR3 and PR3/4 being derived from fusion of PSA4 with R5/3OA.The phenotypic properties of these two lines and their hybrid derivatives are summarised in Table 3:6 and are all consistent with this conclusion.The implications for the relationship between differentiation and metabolic cooperation are difficult to define without further investigation and this is done in the following chapters,in which lines are isolated for their reduced cooperation properties [Chapter 4] and for their reduced differentiation propeties [Chapter 5].It can however be concluded that the PSA4 phenotype is either dominant or semi-dominant in respect to differentiation and metabolic cooperation.

The finding that hybrid lines,especially PR3,become feeder independent when grown in medium containing high serum levels may also indicate a combination of the parental phenotypes in the hybrid.This property is of special interest if it can be shown to be linked to differentiation.

One major feature of both PR3 and Pr3/4 is their heterogeneity,this is reflected both by the wide range of differentiation observed in colonies plated out into STO conditioned medium and found in EB suspensions and the wide range of counts obtained in cooperation experiments.This could be a result of the wide range of chromosome numbers counted which could either demonstrate differential chromosome loss or that the line is made up of hybrids of several

Table 3:5

A SUMMARY OF THE PROPERTIES OF THE EC CELL LINES PSA4 AND R5/30A AND OF THEIR HYBRID DERIVATIVES PR3,PR3/4 AND PR5

Summarises the results of Uridine transfer,differentiation (embryoid body and tumour formation) and plating efficiency (HAT and Ouabain) experiments.

Notes:

i) Difb - differentiation in embryoid bodies (*in vitro*) differentiation

ii) Dift - differentiation in mouse tumours (*in vivo*) differentiation

iii) $\frac{T-NT}{X}$ - median of 'touching' counts minus median of 'non-touching' counts adjusted for ploidy (X).Measures the extent of metabo cooperation.

iv) %age - percentage of contacting cells which have a high degree of cooperation as measured by computor analysis.

v) nd - indicates that the experiment was not done

Cell Line		Ploidy	T-NT/X	%age	Phenotype			
						In Vitro	In Vivo	
PSA4	3a	2	8.2	85				
	3b	2	8.4	82				
	3c	2	10.5	96	Mec +	Difb+	Dift+	HPRT+ Oua^S
	3d	2	6	90				
R5/30A	3a	4	1.1	74.7				
	3d	4	1.5	80	Mec -	Difb -	Dift -	HPRT- Oua^R
	5b	4	3.7	86.3				
PR3	3a	5	5.6	90.7				
	3b	5	9.6	99.7	Mec +	Difb +	Dift +	HPRT+ Oua^R
	3d	5	4.2	95				
PR3/4	3a	5	8.1	98				
	3b	5	7.8	90.3	Mec +	Difb +	nd	HPRT+ Oua^R
	3c	5	2.1	97				
PR5	3a	2	12	93				
	3b	2	16	90.4	Mec +	Difb +	Dift +	HPRT+ Oua^R
	3c	2	3.3	83				
	3d	2	6.3	94				

fusion events which are displaying different properties.This question could be investigated by further isolation of cell lines and by cloning.Alternatively the wide range of counts obtained in uridine transfer experiments could simply be due to the increased ploidy of the line as compared to the diploid mec+ line PSA4 so that the sensitivity of the experiment is increased because grains can be counted up to levels (200+ per cell) that is not possible in the smaller diploid cells.

CHAPTER 4

ISOLATION OF METABOLIC COOPERATION DEFICIENT LINES FROM THE HYBRID EC LINE PR3

The cell line PR3 described in the previous chapter is a sub-hexaploid hybrid line derived from the fusion of the non-differentiating,mec- ec line R5/3OA and the pluripotent,mec+ ec line PSA4.On the basis of this preliminary characterisation the line PR3 was chosen for further analysis,because the remaining hybrid line (PR3/4) was derived from the contamination of one line (PR4) with another (PR3) and therefore was of known heterogenous origin.

PR3 is capable of both spontaneous differentiation and metabolic cooperation and it was hoped,by the isolation of mec- hybrid derivatives, that it would be possible to demonstrate whether these two characteristics are linked or whether they are independent of one another.The selection procedure was facilitated by PR3 being of hybrid origin so that the incidence of spontaneous loss was expected to be high enough to allow the selection variant lines by simply plating out the PR3 cells in a selective medium without the need for the introduction of further mutagen treatment.Because the original line was isolated in HAT and Oua medium PR3 was therefore both resistant to Ouabain and was HPRT+,it was therefore necessary to isolate HPRT- variants of PR3 using thioguanine resistance,before mec- lines could be selected using the "Kiss of Death" method [2.5.2.].The selection of thioguanine derivatives is therefore described in the first part of this chapter,this is then followed by the results of the "Kiss of Death" selections.

4.1. Selection of Thioguanine Resistant Derivatives of the Hybrid EC Line PR3

Cells which are HPRT- can survive in 6-Thioguanine (6-Tg) [63],whereas those cells which have the HPRT enzyme do not survive.Therefore the most simple method of isolating an HPRT deficient cell line is to select for growth in 10-30µg/ml thioguanine.After lines had been selected in this way they were tested for survival in HAT to show that it was the absence of the HPRT enzyme rather than some other mechanism which was making them resistant to 6- Tg.One of the parent lines (R5/3OA) of the hybrid line PR3 was HPRT- and therefore it was expected that the most likely alteration to occur in PR3 cells incubated in

6-Tg would be the loss of the HPRT+ gene carrying chromosome (the X-chromosome),leaving the hybrid line with the HPRT- phenotype of R5/3OA.

4.1.1. PR3 Cell Survival in 6-Thioguanine

Cells were plated out both on STO feeders and in STO conditioned medium at densities of either $3x10^4$ cells per dish [63] or at 10^3 cells per dish [2.5.59],the lower density was added to the protocol as the segregation frequency was not known.

Dishes were then fed with 6-Tg at concentrations of 10µg/ml,20µg/ml or 30µg/ml and then were monitored for colony survival.Figure 4:1 illustrates the colony survival of PR3 cells in 6-Tg both on and off STO feeders.Results show that plating the cells out at cloning density (10^3 cells per dish) resulted in very little cell survival,the survival frequency of 0-1 colonies per dish is consistent with the survival rate of colonies found in those dishes plated at $3x10^4$ cells per dish which is a 30 fold increase in density.

Survival of cells as determined by colony counts was,as would be expected,greater both on and off feeders when 10µg/ml 6-Tg was used compared with 30µg 6-Tg,survival in 20µg was intermediate between the two.This observation was more marked when cells were plated in STO conditioned medium when there was almost a two-fold increase in survival between the higher and lower 6-Tg concentrations ($1.08x10^{-4}$ in 30µg/ml-$1.85x10^{-4}$ in 10µg/ml).The survival of PR3 when plated on STO feeders seemed less dependent on 6-Tg concentration,and the average survival rates of PR3 colonies on feeders was better,although when low concentrations of 6-Tg were used plating the cells in conditioned medium,rather than on feeders,seemed to be of some advantage [Figure 4:1].This effect is not,however,significant because of the extent of overlap of the error bars.

Plating cells out at cloning density in 6-Tg resulted in very little cell survival,the survival of 0-1 colonies per dish is consistent with the segregation frequency of colonies found in those dishes plated at $3x10^4$ cells per dish which is a 30 fold increase in density.

4.1.2. Isolation of Thioguanine Resistant Lines from Colonies of PR3 growing in 6-Tg

72 colonies were picked from the dishes of colonies growing in 6-Tg described in 4.1.1.24 of these from cells plated in 30µg/ml,24 from 20µg/ml dishes and the remaining 24 from 10µg/ml dishes,approximately 25% of these were eventually chosen and grown up into permanent frozen stocks.Those colonies which grew well in 30µg/ml and resembled PR3 in morphology were preferred for permanent isolation as these were expected to be the most likely to be HPRT- segregants.Half of the lines isolated were from dishes containing feeders and the other half from dishes fed with STO conditioned medium [Table 4:1] and they were designated PR3Tg1-20 (excluding PR3Tg15 and PR3Tg19 which were lost through contamination).

4.2. Karyotyping of Thioguanine Isolated Lines

To determine that the lines isolated were of hybrid (PR3) origin and not a result of low level contamination of the PR3 line with the HPRT- parent line R5/30A,all of the 6-Tg resistant lines isolated were karyotyped and lines chosen for further work on the basis of their karyological similarity to the parent line PR3 coupled with an ec morphology.

4.2.1. Chromosome Counts

It was found that those 6-Tg resistant lines which had the lowest range of chromosome counts and a modal or mean count which was the closest to the PR3 line,were all isolated from colonies growing on STO feeders although it is not known whether or not this is of any significance.All of these lines had the two metacentric markers of the parent line [Figure 4:2].

Only one of the karyotyped isolated lines from the 6-Tg selection had a chromosome count which was grossly different from the parent line and this was the clone Tg10 which was isolated in conditioned medium and was found to have a modal chromosome count of 56,no metacentric chromosomes and a fibroblast morphology.This was most likely to be an STO cell contaminant derived from residual STO cells in the PR3 cell culture,it was not however

Table4:1

A SUMMARY OF 6-THIOGUANINE RESISTANT VARIANTS ISOLATED FROM THE HYBRID LINE PR3

Showing the origin of 18 6-thioguanine resistant cell lines isolated from the hybrid PR3.

Con.medium (STO) - lines selected on gelatin in medium conditioned with STO feeder cells

STO feeders - Lines selected on STO feeder layers

Line	ug/ml 6-Tg	Isolation Conditions
PR3.Tg1	10	Cond.Medium (STO)
PR3.Tg2	30	"
PR3.Tg3	30	"
PR3.Tg4	30	"
PR3.Tg5	30	"
PR3.Tg6	30	"
PR3.Tg7	30	"
PR3.Tg8	30	"
PR3.Tg9	30	"
PR3.Tg10	30	"
PR3.Tg11	30	STO feeders
PR3.Tg12	30	"
PR3.Tg13	30	"
PR3.Tg14	30	"
PR3.Tg16	30	"
PR3.Tg17	30	"
PR3.Tg18	30	"
PR3.Tg20	30	"

Figure 4:1

SURVIVAL OF PR3 CELLS AFTER 10 DAYS INCUBATION IN 6-TG

Colony survival of PR3 cells in 6-thioguanine (6-Tg) is illustrated in tabular form showing survival; a) when cells were plated out onto STO feeder cells,and b) when cells were plated onto gelatin treated dishes in the presence of STO feeder conditioned medium All results are presented in graphic form in 4:1(c)

O - conditioned medium

□ - on feeders

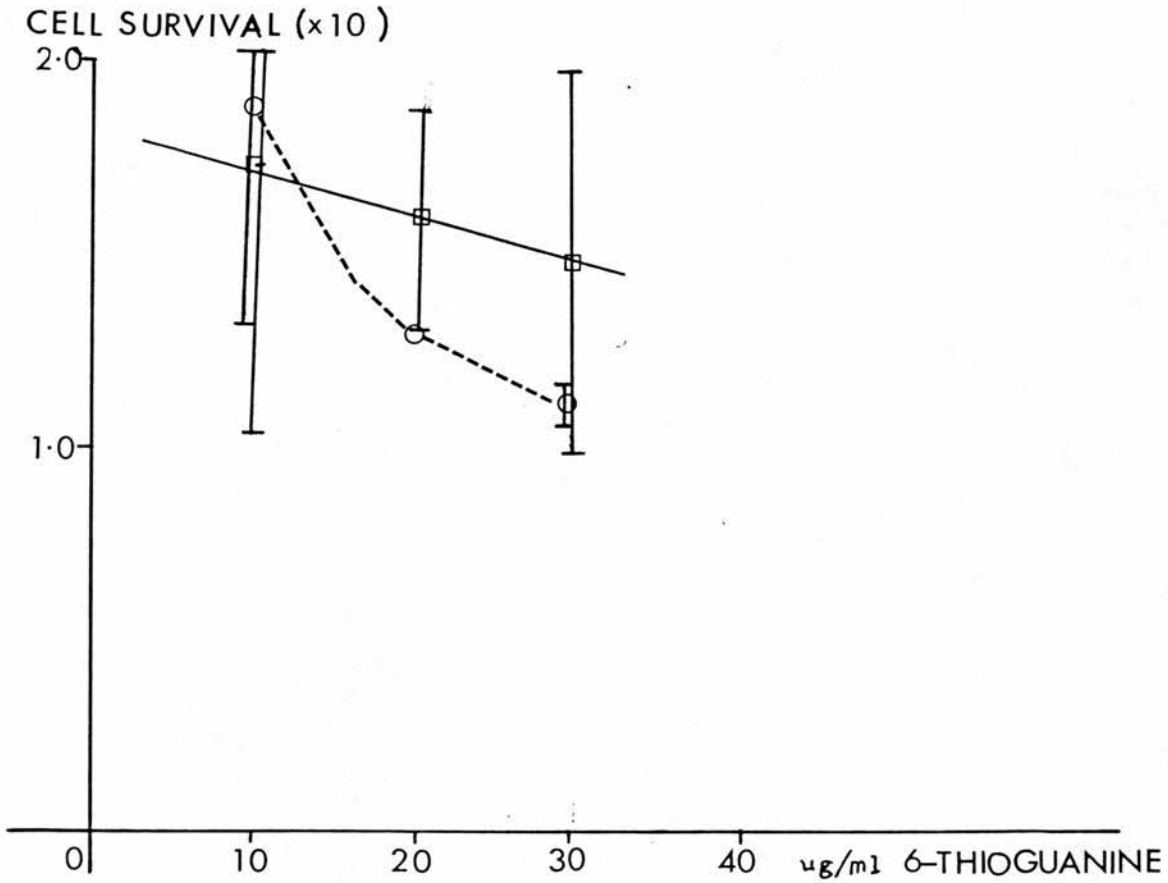
a) On Feeders

concentration of 6-Tg (ug/ml)	survival of colonies $3x10^4$ cells/dish		10^3 cells/dish		survival per 10^3 cells
30	38	43.5	2	1	1.45
	49		0		
20	51	47	-	-	1.57
	43				
10	58	50.5	1	0.5	1.68
	43		-		

b) conditioned medium

concentration of 6-Tg (ug/ml)	survival of colonies $3x10^4$ cells/dish		10^3 cells/dish		survival per 10^3 cells
30	32	32.5	-	-	1.08
	33				
20	38	38	-	-	1.27
10	47	55.5	0	0	1.85
	64				

c)



visible in the karyotypes of either the original parent line or in the other 6- Tg resistant clones and therefore was probably an isolated event.

The lines chosen for the isolation of mec- lines were PR3Tg11,PR3Tg12,PR3Tg13 and PR3Tg20.However,the first successful isolation a mec- line was derived from the line PR3Tg12 and so this line was used in all further experiments.

The karyotype of PR3Tg12 was found to have around 114 chromosomes including 2 metacentric marker chromosomes.Figure 4:3 illustrates these features.

4.3. Characterisation of 6-Tg Resistant Cell Lines

The 6-Tg resistant hybrid lines chosen for use in "Kiss of Death" experiments were further characterised with respect to their morphology and survival in HAT,Oua and 6-Tg containing media.The line PR3Tg12 was cloned and the properties of one of its clones (PR3Tg12.7) also investigated.

4.3.1. Morphology

Like those of the parent line PR3 the cells of the line PR3Tg12 were found to be epithelioid with large nuclei containing 5 or more prominent nucleoli.They were found to be STO feeder dependent and differentiated in the absence of feeders [4.8.1].These cells also,like the parent line,grew in STO conditioned medium.These features are illustrated in Figure 4:4.

4.3.2. Survival of 6-Tg Isolated Lines in HAT,Oua and Thioguanine

Cell lines isolated in 6-Tg should be resistant to the toxic effects of thioguanine but should not be able to grow in the presence of HAT medium if the mode of their 6-Tg resistance is due to the absence of the enzyme HPRT.Cell lines isolated from PR3 in this way should also be Ouabain resistant,but will obviously not now grow in HAT + Oua medium.

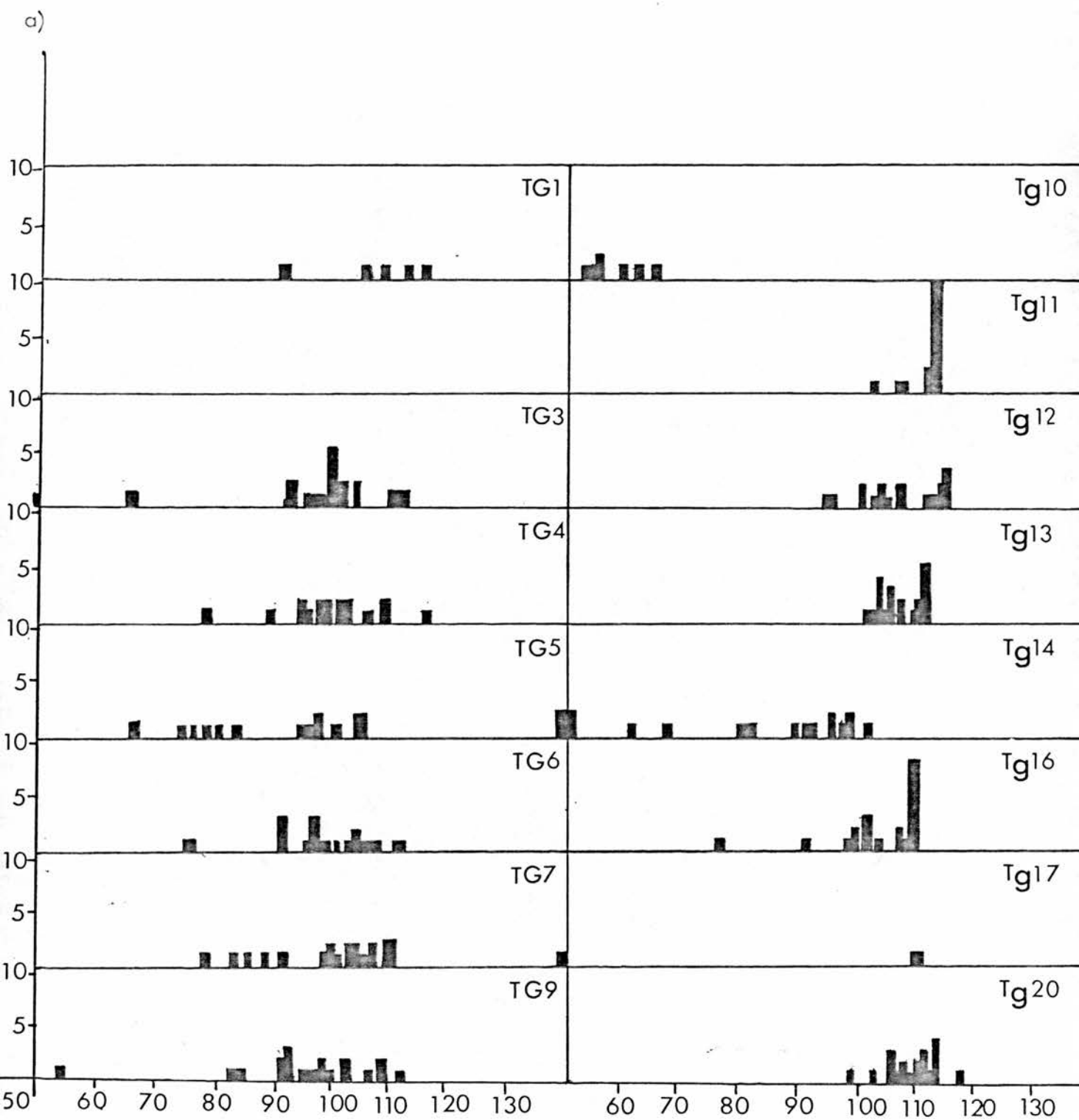
The results of plating efficiency experiments,all carried out in STO conditioned medium,[Figure 4:5a] fulfil these predictions so that of the 5 lines tested

Figure 4:2

ANALYSIS OF CHROMOSOME COUNTS OF PR3 DERIVED 6-TG RESISTANT VARIANTS

a) Histogram analysis of the chromosome counts obtained from counting 20 separate metaphase spreads from slides of colchicine treated cells.Each of the lines shown,with the exception of Tg10 is found to have a subhexaploid karyotype similar to that of the hybrid PR3 from which all variants are derived.

b) Statistical analysis of the chromosome counts illustrated in 4:2(a).The majority of lines are found to have retained the two m metacentric marker chromosomes characteristic of the hybrid line PR3 and of its tetraploid parent line R5 3OA


a)
TG1
Tg10
TG3
Tg11
TG4
Tg12
TG5
Tg13
TG6
Tg14
TG7
Tg16
TG9
Tg17
Tg20
10
5
50
60
70
80
90
100
110
120
130

b)

Cell Line	Mode	Range	Mean	SD	Metacentrics
Tg1	-	92-115	107	8.1	1
Tg3	100	66-112	99.6	9.4	2
Tg4	-	79-116	100	7.5	2
Tg5	-	67-105	89.6	11.8	2
Tg6	-	76-112	99.4	8	2
Tg7	-	79-110	98.8	8.7	2
Tg9	93	55-112	96	12	1-2
Tg10	56	54-66	58.6	4.2	0
Tg11	114	103-114	112.3	3	2
Tg12	116	95-116	108.1	6.5	2
Tg13	112	102-112	107.6	3.5	2
Tg14	-	50-102	83.9	17	0-1
Tg16	110	77-110	104.2	8	2
Tg20	114	99-118	109.9	4.4	2

Figure 4:3

ILLUSTRATION OF THE KARYOTYPE OF THE CLONAL DERIVATIVE]OF PR3TG12;PR3TG12.7

Photograph (x100 objective) of a Leishman's stained preparation of a PR3Tg12.7 metaphase spread obtained by colchicine treatment of a monolayer cultuyre. Arrows indicate metacentric chromosome (Overall magnification = 1024)

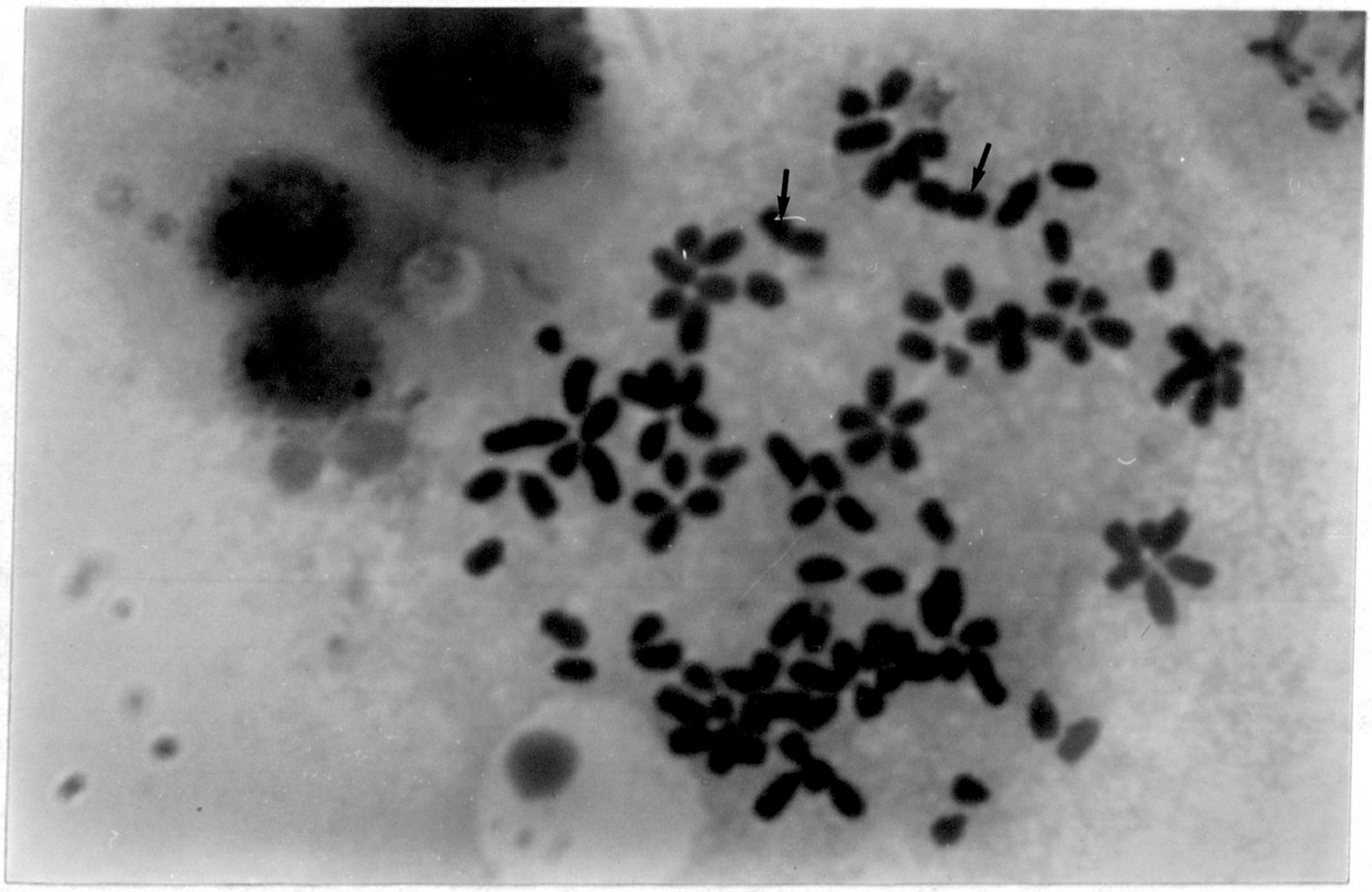


Figure 4:4

LIGHT MICROSCOPE MORPHOLOGY OF PR3TG12 CELLS IN MONOLAYER

Shows the morphology of growing PR3Tg12 cells in monolayer culture. Cells are epitheliod with large nuclei and prominent nucleoli (arrowed).Fibroblast cells are the STO feeders. Magnification = 1024

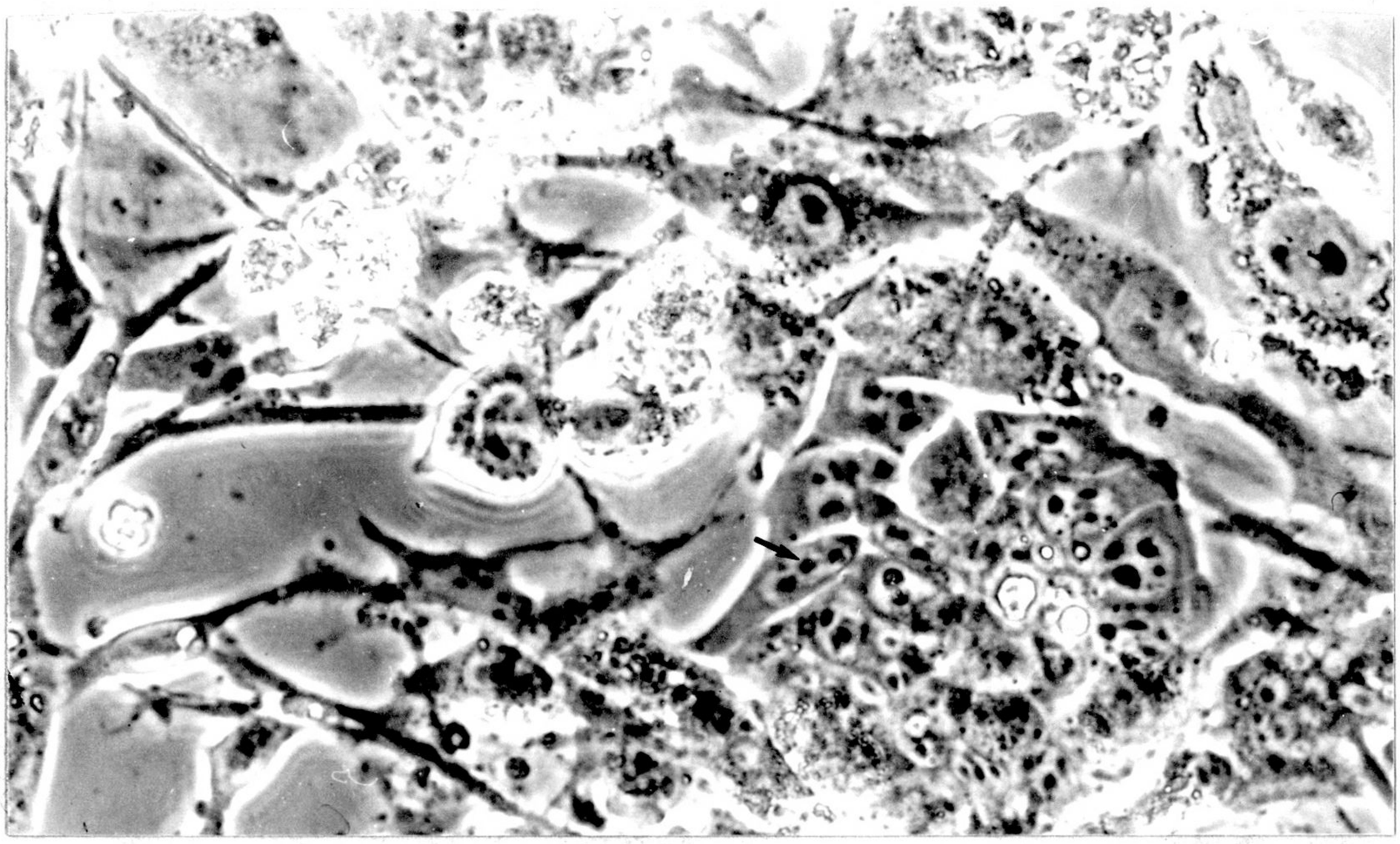


Figure 4:5

PLATING EFFICIENCIES OF LINES SELECTED IN 6-TG AND PARENT LINES IN HAT,OUABAIN AND THIOGUANINE

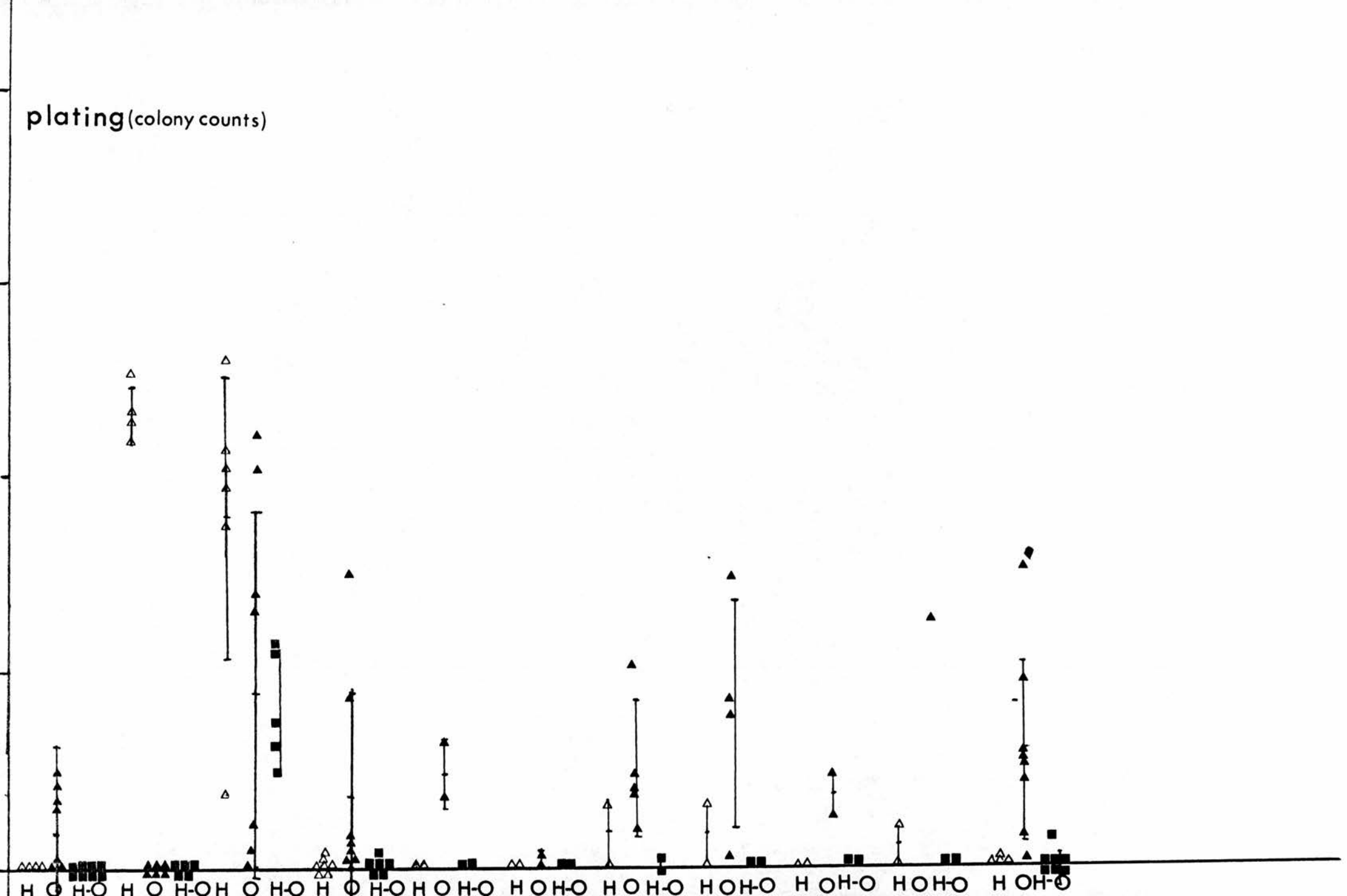
a) Shows the results of plating out PR3 and its thioguanine derived variants into medium containing HAT,Ouabain or both.As was expected the 6-Tg selected lines PR3Tg12,PR3Tg1,PR3Tg6,Pr3Tg11 and PR3Tg14 and the "Kiss of Death" selected lines Kd1a,Kd1a.6 and Kd11Ba are all sensitive to HAT and therefore do not grow in either HAT or HAT + Oua medium.

△- HAT medium
▲- Oua medium
■- HAT+Oua medium

b) Illustrates thioguanine resistance in the lines R5 3OA,PR3Tg12, Kd11Ba,Kd1a.6,Tg6 and Tg1.Lines which do not grow in medium containing this drug are PR5,PR3 and PSA4.

Note: The "Kiss of Death" selected lines Kd1a,Kd1a.6 and Kd11Ba are discussed in section 4:4 of this chapter.

plating (colony counts)



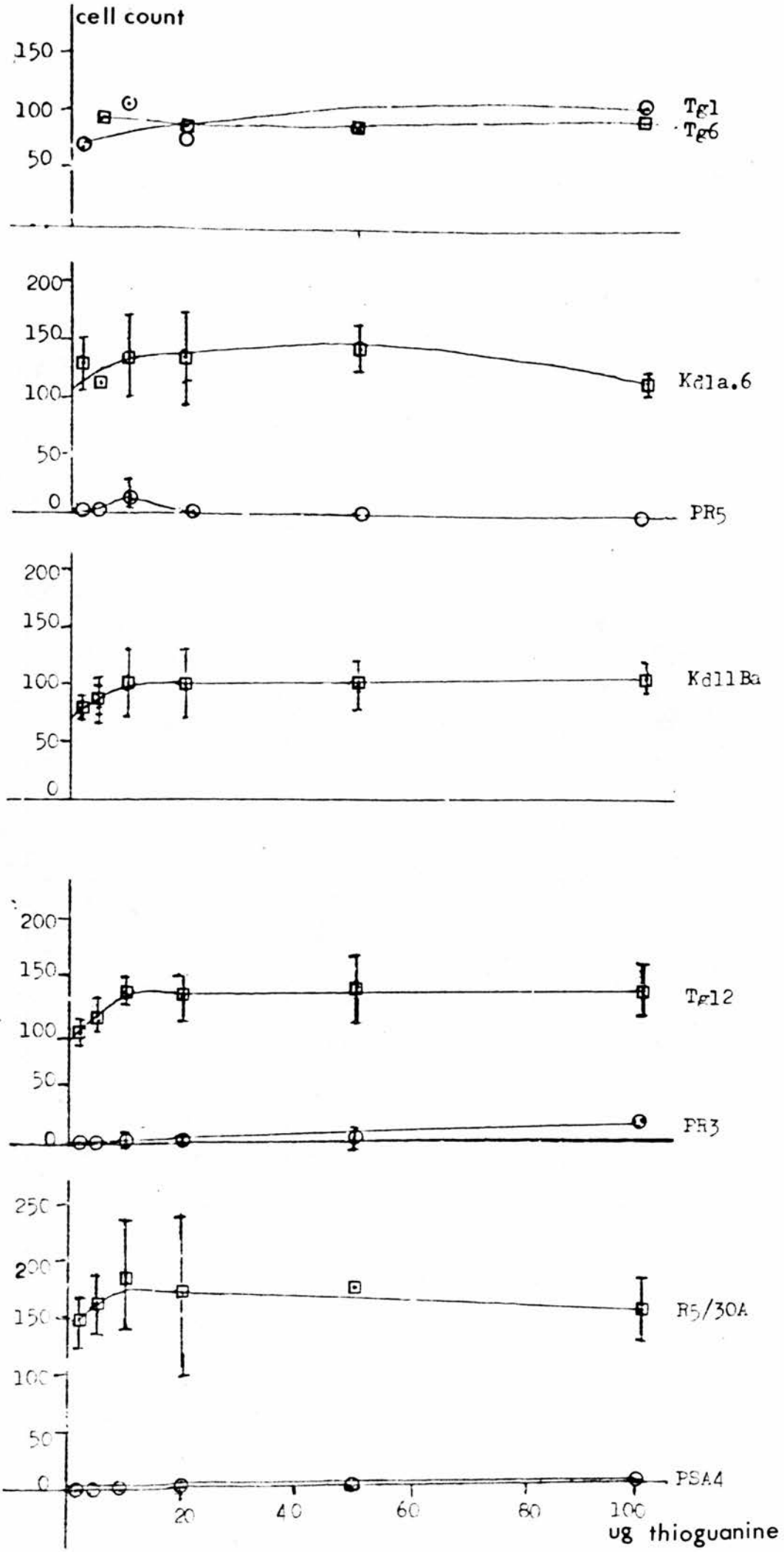

cell count
150
100
50
Tg1
Tg6
200
150
100
50
0
Kd1a.6
PR5
200
150
100
50
0
Kd11Ba
200
150
100
50
0
Tg12
PR3
250
200
150
100
50
0
R5/30A
PSA4
20
40
60
80
100
ug thioguanine

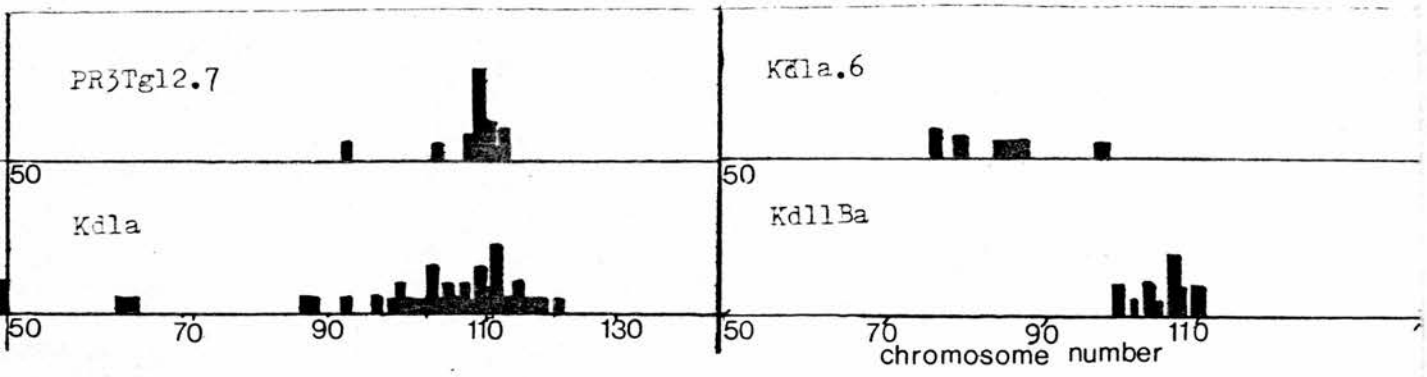
Figure 4:6

KARYOTYPE OF VARIANT HYBRID LINES DERIVED FROM PR3TG12

a) Histogram presentation of chromosome counts of 20 separate metaphase spreads of Leishman's stained slides of colchicine treated monolayer cultures.

b) Table of statistical analysis of the results illustrated in 4:6 (a).

a)



b)

Cell Line	Mode	Range	Mean	SD	Metacentrics
Kdla	112	50-120	102.5	16.3	2
Kdla.6	-	78-99	85.8	6.5	1
Kdl1Ba	108	101-111	106.4	3.2	2
Tgl2.7	110	93-103	109.6	4.8	2

(PR3Tg12,PR3Tg1,PR3Tg14,PR3Tg11 and PR3Tg6) all grew in medium containing Ouabain but not in HAT or HAT + Oua medium.In comparison the hybrid line PR3 grew in all three types of medium but the hybrid parent lines PSA4,which is HPRT+,and R5/3OA which is resistant to Ouabain,grew in one type of medium only (HAT medium and Oua medium respectively).

Similarly the survival of the lines PR3Tg1,PR3Tg6 and PR3Tg12 in various concentrations of 6-Tg paralleled that of the line R5/3OA in contrast to the plating efficiencies of the lines PR3,PR5 and PSA4,all of which showed none or very little survival at all under these conditions [Figure 4:5b].The survival of the hybrid derived lines seems to be greater at higher concentrations of 6- Tg (50-100µg/ml) than that of the parent line R5/3OA although with this data it is not possible to show whether or not this is statistically significant.This may also be due to non-specific toxic effects of the STO feeder cells as it has previously been shown that the presence of STO feeder cells can allow survival of R5/3OA cells under conditions toxic to them in the absence of these cells (i.e.in the presence of overlaid filter paper in replica plating techniques).

These hybrid derived thioguanine resistant cell lines therefore have a phenotype like that of R5/3OA with respect to their performance in the presence of HAT,Oua and 6-Tg and so can be considered to be both Oua^r and HPRT- and therefore suitable for the isolation of mec- derivatives using the "kiss of Death" method.

4.3.3. Cloning of PR3Tg12

In order to determine the stability of the PR3Tg12 karyotype this line was cloned.One of these clones,PR3Tg12.7,was karyotyped [Figure 4:6] and was shown to have the two metacentric marker chromosomes and a modal chromosome count of 110 with all counts falling within a very small range.Figure 4:3 illustrates these features which compare well with those of PR3Tg12 [Figure 4:3] with a modal chromosome count of 116 and PR3 whose modal count is 100 [Chapter 3],both of these lines having the metacentric chromosomes.

The very narrow range of chromosome counts found in this clonal line is an indication that the karyotype of the hybrid has stabilised and could indicate that the wide ranges of chromosome counts found in the uncloned lines

(PR3,PR3Tg12) are due to heterogeneity within the population rather than to continuos chromosome loss.

4.4. Selection of Cooperation Deficient Lines from 6-Tg Resistant Hybrids

Cell lines were isolated using "Kiss of Death" selection [2.5.2] on co- cultures of PR3 and PR3Tg12 to isolate two cell lines by picking surviving colonies [Table 4:2].One of these lines (Kd1a) was then used in a "kiss of Death" selection and another 5 lines isolated [Table 4:2].Two of these putative cooperation deficient lines,Kd11Ba and Kd1a,were karyotyped and further characterised.The results of these investigations are the subject of the rest of this chapter.

4.4.1. Cloning of Kd1a

The line Kd1a was cloned in order to determine whether all of its characteristics could be inherited by separate clones or whether the behaviour of Kd1a was due to a combination of the characteristics of the population of cells making up Kd1a.One of the clones Kd1a.6 was therefore karyotyped and characterised in parallel with the Kd1a and Kd11Ba lines and the results of these investigations are reported in the sections following which deal with the characterisation of these lines.

4.5. Morphology of Lines Selected by "Kiss of Death"

Like other lines derived from PR3 and described in previous chapters,the lines Kd1a,Kd1a.6 and Kd11Ba are epithelioid cells with large nuclei and several (4 or more) prominent nucleoli.The cells are STO feeder dependant and grow in close association with each other in monolayer culture [Figures 4:8].However these lines do appear to be slightly less tightly packed in culture than the previous lines (PR3 and its derivatives) and are easily distinguishable from PSA4 [Figure 3:4,Chapter3],their morphology in monolayer culture becoming now closer to that of R5/3OA [Figure 3:4,Chapter 3].

Table 4:2

ENRICHMENT OF PR3TG12 FOR COOPERATION DEFICIENT CELLS
USING 6-THIOGUANINE "KISS OF DEATH"

Illustrates the variant lines isolated from cocultures of PR3 and PR3Tg12 grown in 6-Thioguanine.Cooperation deficient cells are selected for as PR3 cells and PR3Tg12 cells cooperating with PR3 cells are killed by 6-Tg (30μgml.)

Round 1 Selection : PR3/PR3Tgl2 Co-Culture

Kd1a
Kd1b

Round 2 Selection : PR3/Kd1a Co-Culture

Kd11Ba
Kd11Ca
Kd11Da
Kd11Ea
Kd11Fa

4.6. Karyotyping of Kd Lines

Chromosome counts of the lines Kd1a,Kd1a.6 and Kd11Ba are illustrated in Figure 4:6.Like the parent lines the range of counts is quite large,with the exception of the cloned line Kd1a.6,but the mode is still in the sub-hexaploid range in the lines Kd1a and Kd11Ba,although the clonal line Kd1a.6 has lost a lot of chromosomes,including one of the metacentric markers,and its mean chromosome number is close to being tetraploid,although higher than that of R5/3OA.Figure 4:7 illustrates typical leishman stained karyotypes of these lines.

4.7. Plating Efficiency of KD Lines in HAT,Oua and Tg

Because the lines Kd1a,Kd1a.6 and Kd11Ba were all expected to have derived from the 6-Tg resistant derivative of PR3 (PR3Tg12) in the "Kiss of Death" experiments.They were expected to have the same phenotype (Oua^r and HPRT-) as that found both in PR3Tg12 and in R5/3OA.They are also,therefore,obviously expected to be resistant to the toxic effects of 6- Tg.

4.7.1. Growth of Putative mec- Lines in HAT and Oua

Results [Figure 4:5a] confirm these predictions and show that in contrast to PR3 and PSA4,but like PR3Tg12 and R5/3OA the cell lines Kd1a,Kd1a.6 and Kd11Ba all survive in Ouabain but cannot survive in medium containing HAT.As with previous experiments the survival of these cell lines in Ouabain is variable.This is discussed in further detail in Chapter 3 [3.6].

4.7.2. Growth of Putative mec- Lines in 6-Tg

Similarly,the growth of the lines Kd1a,Kd1a.6 and Kd11Ba reflects their origin [Figure 4:5b] from the 6-Tg resistant line PR3Tg12 so that their survival profiles were like those of R5/3OA and the PR3Tg lines in contrast to the profiles of PR3,PR5 and PSA4 which do not survive in thioguanine at all.

Figure 4:7

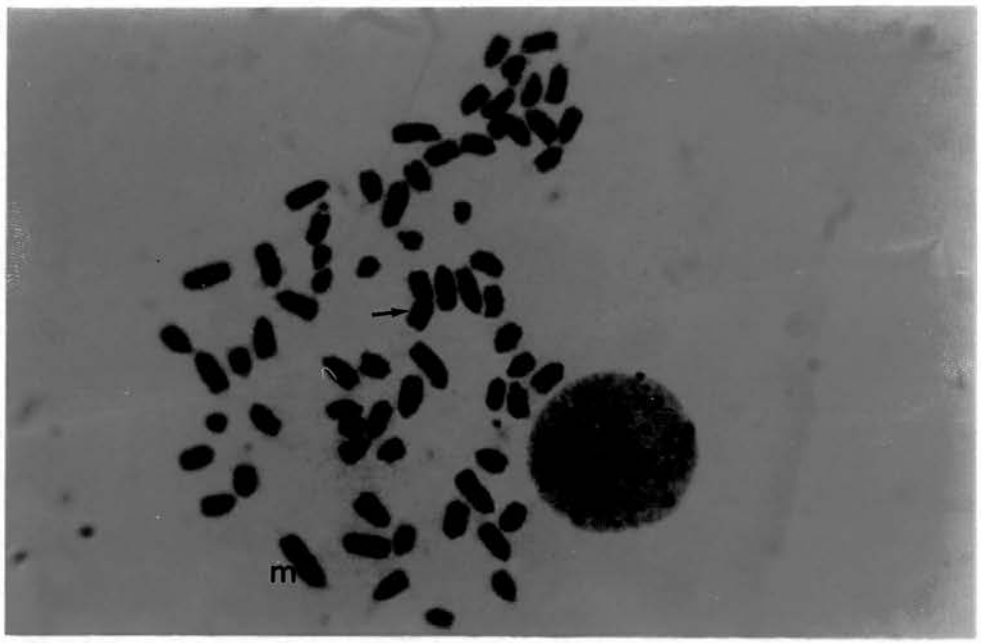
ILLUSTRATION OF THE KARYOTYPE OF VARIANT LINES ISOLATED FROM PR3TG12

Leishman's stained preparations of metaphase chromosomes prepared by colchicine treatment of monolayer cultures.Arrows indicate metacentric chromosomes.

a) Metaphase spread of Kd1a.6 (magnification 5000) showing one metacentric chromosome and a chromosome count of 70.(m) indicates a marker chromosome which has a heterochromatic region halfway down the long arm.

b) Metaphase spread of Kd11Ba (magnification 4000) with a chromosome count of 110,two metacentric chromosomes and one marker chromosome (m).

a



b

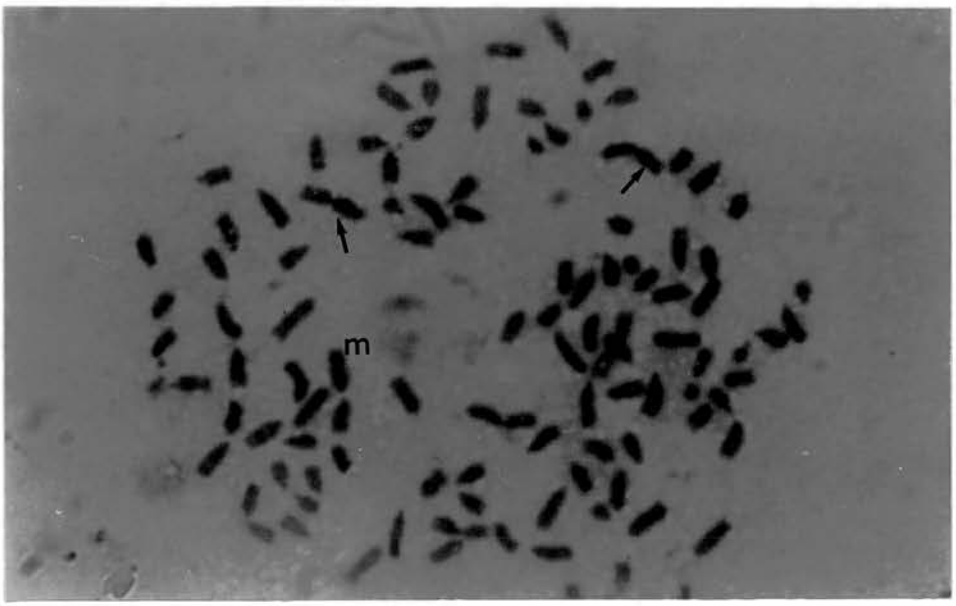


Figure 4:8

MORPHOLOGY OF HYBRID DERIVED VARIANTS ISOLATED FROM PR3 BY THIOGUANINE "KISS OF DEATH"

a) Illustration of the morphology of monolayer cultures of the line Kd1a.6 when grown on an STO feeder layer and viewed under phase contrast on an inverted microscope.

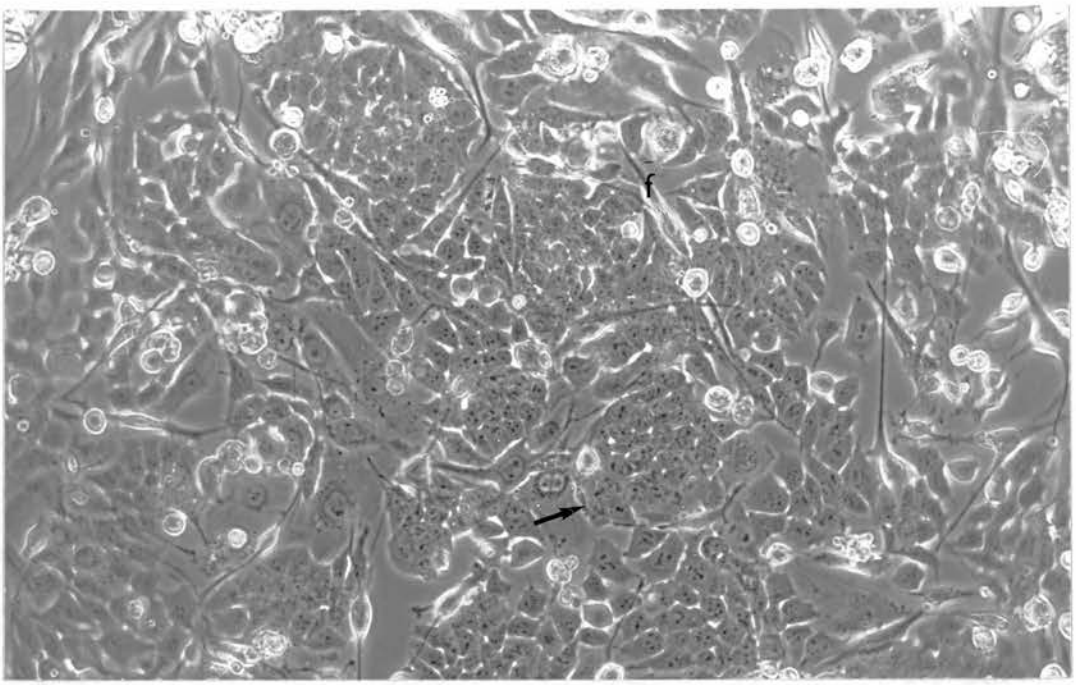
i) Low power (magnification 320)
ii) High power (magnification 1024)

b) Illustration of the morphology of a monolayer culture of the variant Kd11Ba when grown on an STO feeder layer and viewed under phase contrast on an inverted microscope.

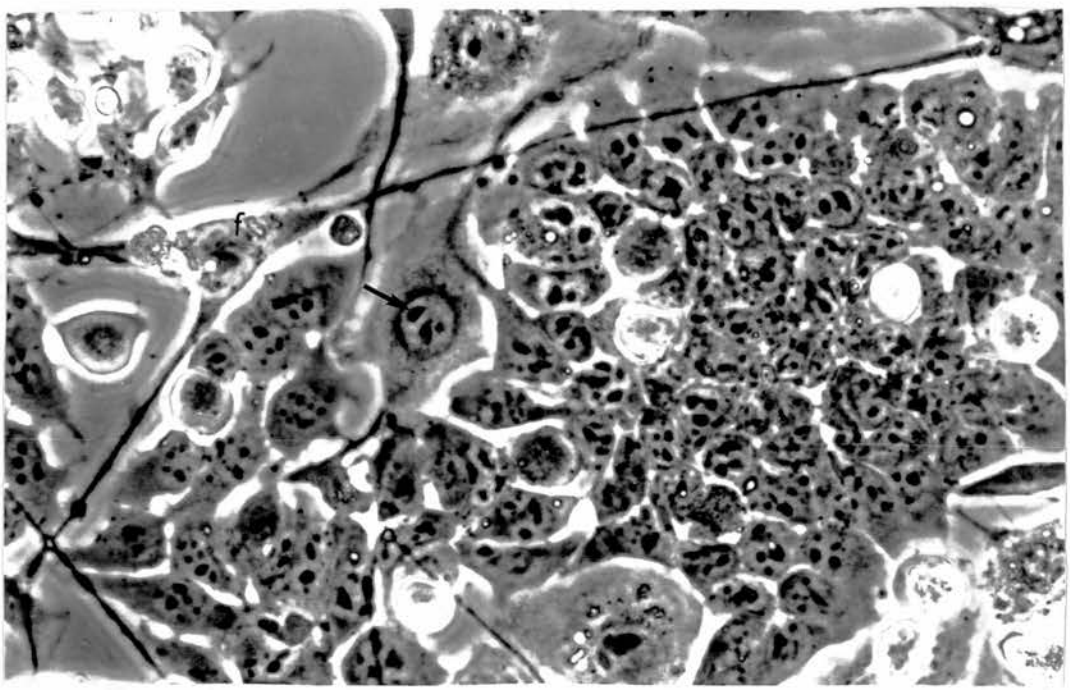
i)Low power (magnification = 320)
ii)High power (magnification = 1024)

Both hybrid variants shown here have an ec-like epitheliod morphology with large nuclei and several prominent nucleoli. Fibroblast cells with refractile nuclei are STO feeder cells (f). Arrows in **a**i and **b**i indicate ec colonies and arrows in **a**ii and **b**ii indicate nucleoli in the nucleus of an ec cell.

ai

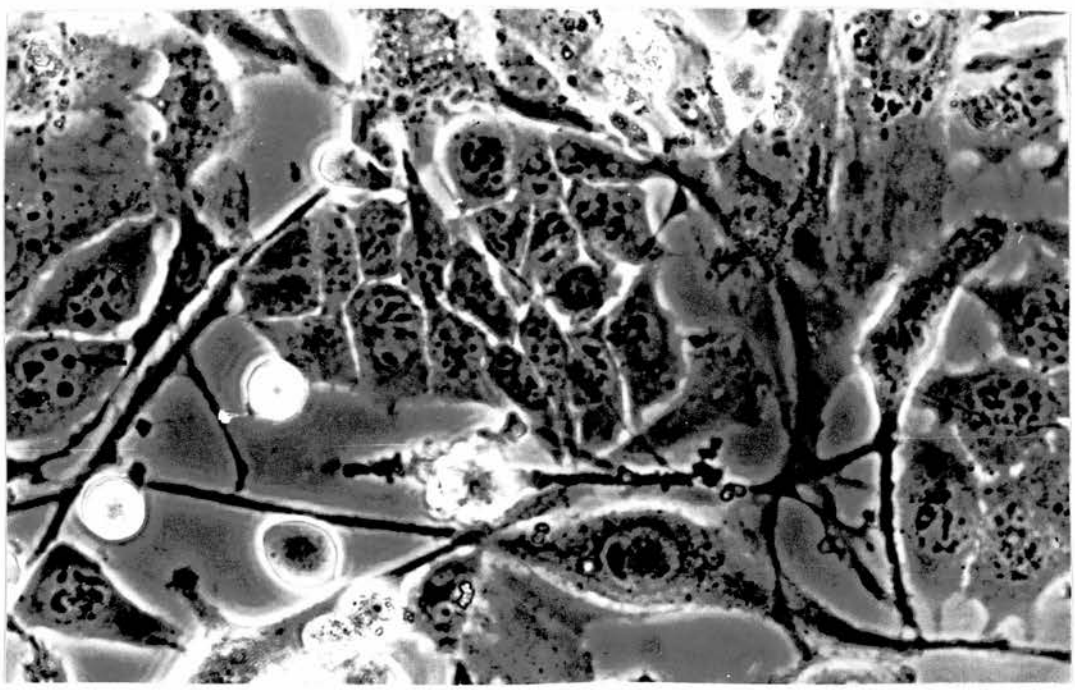


aii



bi

bii



4.8. Differentiation Ability of Cell Lines Derived from PR3

As described in Chapter 3 [3.5],the hybrid derivative (PR3) of PSA4 and R5/3OA is capable of forming differentiated tissues both *in vitro*,in embryoid body suspension,and *in vivo* in teratocarcinomas formed at the injection site,when syngenic mice were injected with cell suspensions.It was also observed that PR3 colonies showed variable differentiation when plated into STO conditioned medium.Thioguanine resistant variants and variants selected by kiss of death were therefore tested for their ability to differentiate as measured by these three criteria.

4.8.1. Differentiation *In Vitro*

It was observed that,like the parent line PR3,colonies of both the 6-Tg resistant lines and the Kd lines displayed heterogeneity in the extent of their differentiation when they were plated onto gelatin in STO conditioned medium and this is further discussed in Chapter 5.

Analysis of the extent of differentiation of these cell lines in embryoid body (EB) suspension [2.4.5] has revealed that all of the lines Kd11Ba,Kd1a.6 and PR3Tg12,were able to form differentiated tissues when cultured in EB suspension [Figure 4:9] but that when the extent of this differentiation was compared to the hybrid line PR3 from which these lines were all derived,all (including PR3Tg12) showed a slightly reduced ability to differentiate.

However,in comparison to the results obtained with embryoid bodies derived from R5/3OA which were very poorly differentiated,all three lines (PR3Tg12,Kdla.6 and KdllBa) were clearly differentiating.

4.8.2. Differentiation *In Vivo*

Differentiating tumours [Table 4:3] were obtained at the site of injection in mice injected with cells from the lines Kd1a.6 and from Kd11Ba but none of the mice injected with PR3Tg12 cells produced tumours in this experiment.A further experiment involving the injection of PR3Tg12 [Chapter 5] into further mice

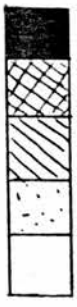
Figure 4:9

EMBRYOID BODY ANALYSIS OF *IN VITRO* DIFFERENTIATION OF THE PR3 DERIVED VARIANTS PR3TG12,KD1A.6 AND KD11BA

a) Actual numbers of Embryoid bodies counted in each of the five categories from 1 (undifferentiated) to 5 (extensive endoderm diff- erentiation and cavity formation).The counts are totaled for each category.

b) Pie-chart presentation of the extent of differentiation in embryoid bodies of the lines PR3Tg12,Kd1a.6 and Kd11Ba as determined by the counts shown in (**a**).Each of the five categories shown (see key) correspond to one of the five categories in (**a**).

Key:



- Undifferentiated
- VE,PE (+)
- VE,PE (++)
- VE,PE,cavity formation (+)
- VE,PE,cavity formation (++)

a)

Cell line		Undifferentiated	VE + PE	VE++ PE	VE PE cavity +	VE PE cavity ++
Tg12		30	63	119	15	1
		21	39	112	14	5
		75	176	239	17	7
		63	234	237	22	5
	total	189	512	707	68	18
Kd1a.6		21	94	175	31	3
		17	98	230	32	4
		24	101	161	37	3
		22	115	196	39	3
	total	84	408	762	139	13
Kd11Ba		24	103	61	11	-
		31	71	50	12	-
		27	85	87	4	-
		36	111	120	5	1
	total	118	370	318	32	.1

b)

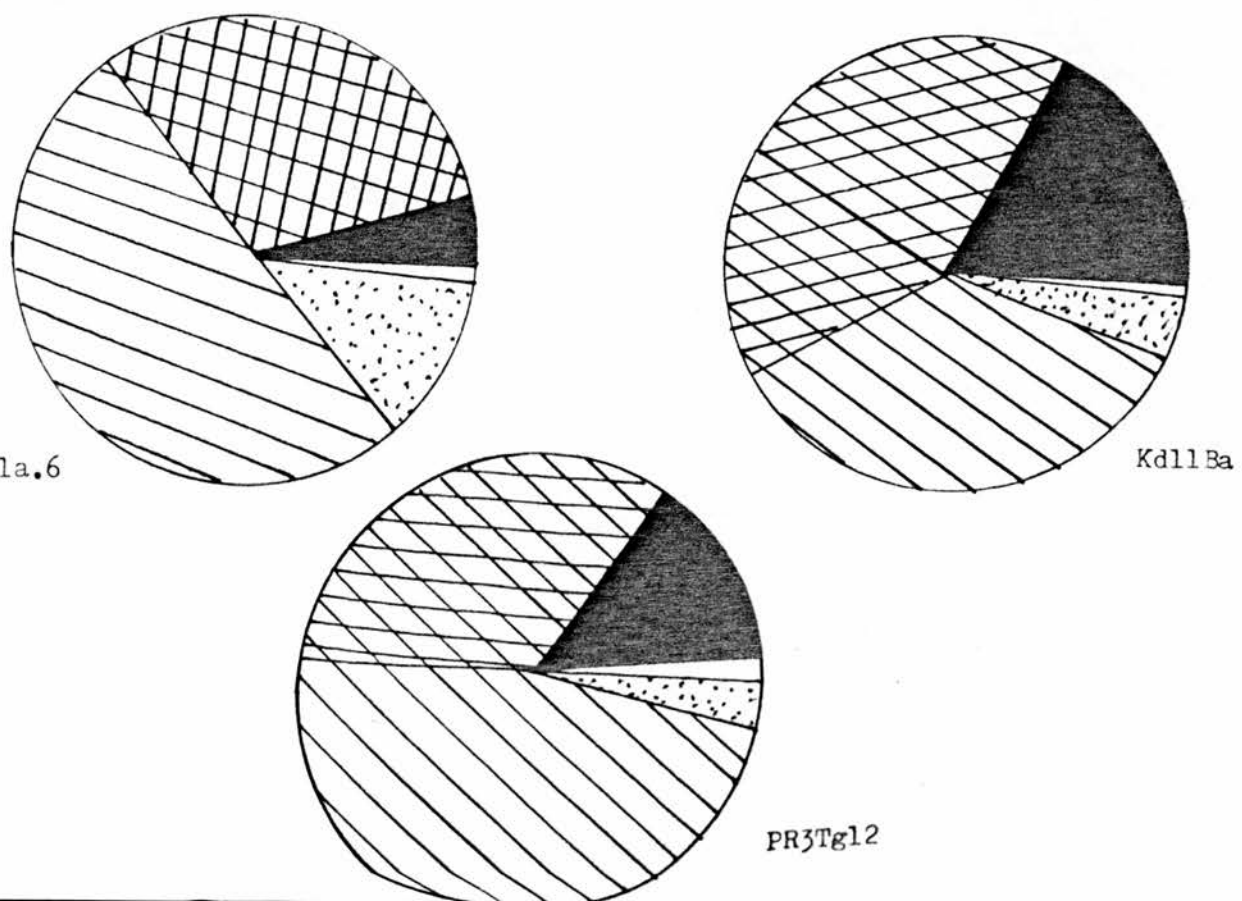


Table 4:3

TUMOUR FORMATION OF PR3 DERIVED HYBRID LINES ON INJECTION INTO SYNGENIC MICE

Shows the result of left shoulder subcutaneous injection of samples of cells from the lines PR3Tg12,Kd1a.6 or Kd11Ba in syngenic (129) mice.The length of time taken to form the tumour is taken as the number of days between injection of the mouse and sectioning of the tumour.

Two experiments were set up Experiment 1 : included tumours from cell lines described in chapter 3 (see Table 3:4).Two male and two female mice were injected for each cell line tested.

PR3Tg12	0/4 tumours formed
Kd1a.6	4/4 tumours formed
Kd11Ba	1/4 tumours formed

All animals remaining after 11 months without forming tumours were killed and autopsied for abnormalities.No further tumours were found. Experiment 2 : Included tumours from cell lines reported in chapter 5 (Table 5:2).

PR3Tg12	2/3

animals not forming tumours were killed and autopsied after 9 months.

Key:

F - female mouse
M - male mouse
N A - not applicable
n.o.ab - no other abnormality found after autopsy

	Cell Line	Passage number	Sex of animal	Days to form	Remarks
1)	PR3Tg12	8	F	N/A	no tumour after 107 days
		8	F	N/A	no tumour after 166 days
		8	M	N/A	no tumour
		8	M	N/A	no tumour
	Kd1a.6	4	F	28	2-3 cm tumour Cystic dysplasia of the uterus
		4	F	34	2-3 cm tumour n.o.ab
		4	M	52	3 cm tumour with a good blood supply Obese animal
		4	M	74	2-3 cm tumour with good blood supply obese animal
	Kd11Ba	3	F	25	3-4 cm tumour with large blood supply n.o.ab
		3	F	N/A	no tumour
		3	M	N/A	no tumour
		3	M	N/A	no tumour
2)	PR3Tg12	13	M		2 cm tumour with good blood supply n.o.ab
		13	M		3cm tumour with good tumour n.o.ab
		13	F		no tumour

yielded a tumour incidence of 2/3 mice injected.

Tumours derived from cells of the thioguanine resistant PR3 derivative PR3Tg12 were found to be predominantly of undifferentiated ec cell type.Small areas of differentiated tissue were also found in these tumours which contained both smooth and striated muscle.Some tumours also had areas of cartilage [Table 4:4].This finding means that the hybrid derived line PR3Tg12 behaves in a very similar way to the R5/3OA parent in its differentiating capacity in mouse tumours and unlike the differentiating parent line PSA4 and the original hybrid PR3 [3.6].

In order to determine the stability of hybrid lines *in vivo* a slice of one tumour,derived from a mouse injected with PR3Tg12,was explanted into a tissue culture dish and incubated in CM(10) [2.5.5].outgrowth cells grew rapidly from the tumour and spread across the dish.They were large epithelioid cells with large nuclei and several prominent nucleoli and resembled the hybrid ec cell lines from which the tumour was thought to be derived.These cells were frozen down into a permanent stock (PR3Tg12TX) and karyotyped.Further characterisation of this line is reported in 4.8.3 and 4.9.1.

Tumours derived from the cloned line Kd1a.6 and from the line Kd11Ba were also found to be largely undifferentiated [Table 4:4] containing predominantly ec cells.Sections from a tumour derived from Kd11Ba cells also had small areas of cartilage,smooth muscle and striated muscle.Smooth muscle was found in all tumours derived from Kd1a.6 cells and striated muscle and cartilage were also found in small quantities in most Kd1a.6 tumours.

The tissue types found in mouse tumours derived from the hybrid variant lines PR3Tg12,Kd1a.6 and Kd11Ba are summarised in Table 4:4.

4.8.3. Karyotyping of PR3Tg12TX

The karyotype of PR3Tg12TX,illustrated in Figure 4:12,reveals a modal count of 111 chromosomes per metaphase spread,but a mean of only 91.8 indicating some *in vivo* chromosome loss or selection for the lower end of the range.Some cells also seem to have lost one of the metacentric marker chromosomes.However this result does illustrate that a tumour caused by

Table 4:4

SUMMARY OF THE TISSUE TYPES FOUND IN MOUSE TUMOURS DERIVED FROM PR3TG12,KD1A6 AND KD11BA

Shows the result of scoring mouse tumours for the presence or absence of eleven categories of differentiated tissue and also for presence or absence of undifferentiated ec cells.As can be seen from the table the predominant cell type in all three types of tumour was undifferentiated ec cells.

1 - Cartilage
2 - Bone
3 - Haematopoiesis
4 - Putative trophectoderm
5 - Neural tube formation
6 - Glandular epithelium
7 - Keratinising epithelium
8 - Ciliated epithelium
9 - Smooth muscle
10 - Striated muscle
11 - Columnarepithelium
(12 - embryonal carcinoma cells)

notes:

i) plus signs in brackets - (+) - indicate that this category is present in some,but not all tumours scored

ii) Double plus signs - (++) - indicate that the cell type (ec) is the predominant tissue in that tumour

iii) Plus signs - + - indicate presence and minus signs - indicate absence of a tissue type

Cell Line	1	2	3	4	5	6	7	8	9	10	11	(12)
R3Tg12	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
	-	-	-	-	-	-	-	-	+	+	-	++
	(+)	-	-	-	-	-	-	-	+	+	-	++
dllBa	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
dla.6	-	-	-	-	-	-	-	-	+	+	-	++
	-	-	-	-	-	-	-	-	-	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	-	+	-	++
	(+)	-	-	-	-	-	-	-	(+)	+	-	++

Table 4:5

A SUMMARY OF THE PHENOTYPE OF 6-TG RESISTANT AND "KISS OF DEATH" SELECTED HYBRID CELL VARIANTS DERIVED FROM PR3

Shows the collated results of tritiated Uridine transfer experiments and the phenotype of each of the lines PR3Tg12,PR3Tg12TX,Kd1a,Kd1a.6 andKd11Ba as determined by the characterisation of these lines presented in this chapter.

Cell line		Ploidy	T-NT/X	%age	Phenotype			
						In Vitro	In Vivo	
PR3Tg12	4a	5	3.6	95.4	Mec+	Difb+	Dift-	HPRT- Oua
	5b	5	9.2	99.2				
	4b	5	5.1	98.7				
PR3Tg12Tx	4a	5	3.6	95.4	Mec+	nd	nd	nd nd
	4b	5	3.3	96				
Kd1a	4a	5	1.5	73.3	Mec-	nd	nd	HPRT- Oua
	4b	5	1.7	84.6				
Kd1a.6	4a	5	1	71.2	Mec-	Difb+	Dift-	HPRT- Oua
	4b	5	2.2	87.9				
Kd11Ba	4a	5	8.8	95.5	Mec±	Difb+	Dift-	HPRT- Oua
	4b	5	1.1	73.7				

nd : not done
difb : differentiation in embryoid bodies (in vitro)
dift : differentiation in tumours (in vivo)
NT/X : median of 'touching' cells minus 'non-touching' cell grain counts adjusted for ploidy (X)
%age: percentage of cells showing a high level of cooperation as calculated by computor analysis

injection with a subhexaploid hybrid (PR3Tg12) can generate a population of subhexaploid cells *in vivo*.

4.9. Metabolic Cooperation

The cell lines described in this chapter were assayed for the extent of their metabolic cooperation ability in autoradiography experiments using tritiated Uridine [2.4.4].

Two experiments are described here,both are part of two separate larger experiments (27/3 and 9/4) whose results are divided between chapters 3,4 and 5 and summarised in chapter 5.In addition to this a third experiment involving the PR3 derived line PR32Tg12 is also presented.

4.9.1. Metabolic Cooperation of Thioguanine Resistant Derivatives of PR3

PR3Tg12,the 6-Tg resistant derivative of the hybrid line PR3 has been shown to be capable of gap junction-mediated communication [Figures 4:11a,4:11b and 4:11c and summarised in Table 4:5) in three experiments.T-NT/X values vary from 3.3-12.8 with a mean of 6,while the percentage cooperation estimate ranges from 93-100%.

These figures are comparable to those obtained for the mec+ parental line PSA4 [3.4.1] in the same experiments (27/3 and 9/4) and are slightly higher than those obtained for the hybrid lines PR3 and PR3/4.

As described previously [4.8.2] the line PR3Tg12TX was derived from a tumour explant from a mouse injected with PR3Tg12 cells.This line also,has been shown to be capable of metabolic cooperation in 2 experiments [Figures 4:11a + 4:11b) with T-NT/X values ranging from 2.8-3.8 (mean 3.2) and percentage cooperation estimates from 94.6-98.6.This result,although lower than those obtained with PR3Tg12,is consistent with results found with the hybrid lines PR3 and PR3/4 and suggests an intermediate cooperation ability.It is higher than results obtained with the R5/3OA Mec- parent line or the hybrid,"Kiss of Death" derived,mec- line Kd1a.6 [4.9.2].

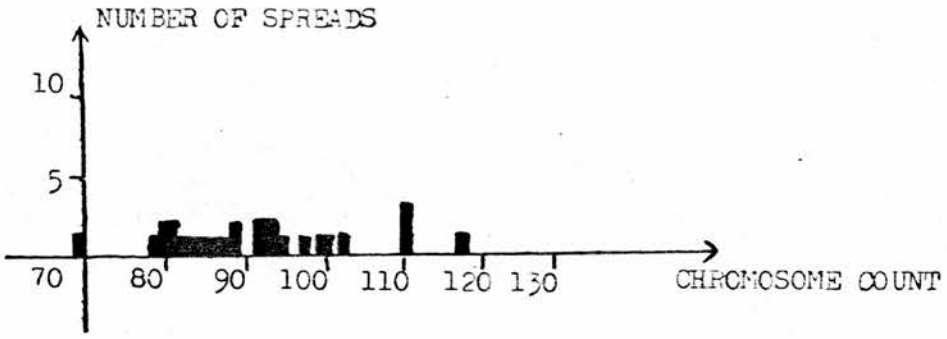
Figure 4:10

ANALYSIS OF THE CHROMOSOME CONTENT OF THE VARIANT PR3TG12TX DERIVED BY ISOLATION FROM A MOUSE TUMOUR CAUSED BY INJECTION OF PR3TG12 CELLS

a) Results of counting Leishman's stained metaphase spreads (30 in all) from slides of colchicine treated PR3Tg12TX cells.

b) Statistical analysis of the chromosome counts illustrated in **a)**.

a)



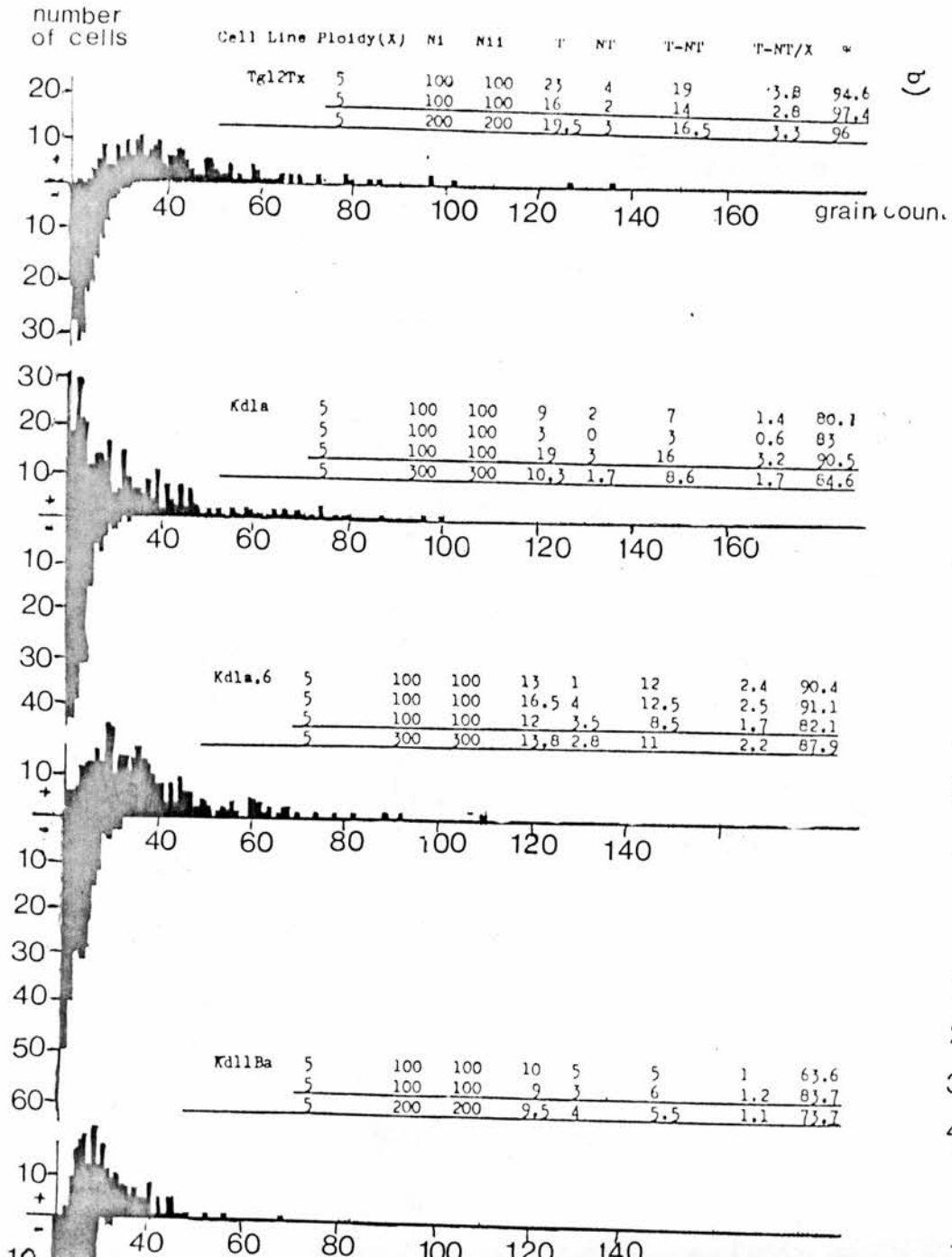
b)

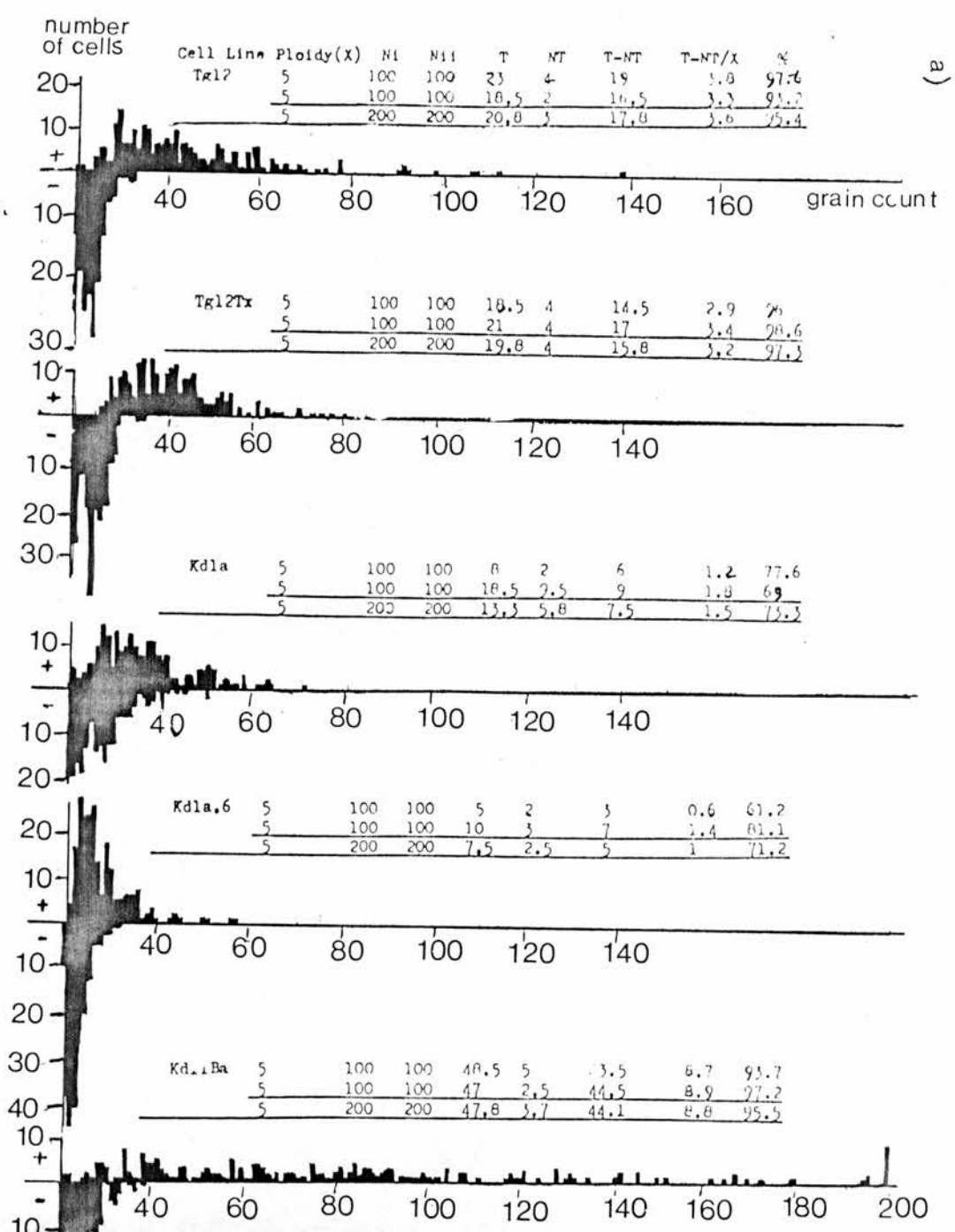
Cell Line	Mode	Mean	Range	Metacentrics	SD
PR3Tg12TX	111	91.8	70-118	1-2	10.9

Figure 4:11

TRITIATED URIDINE TRANSFER ANALYSIS OF THE METABOLIC COOPERATION OF CELL LINES DERIVED FROM THE HYBRID EC CELL LINE PR3

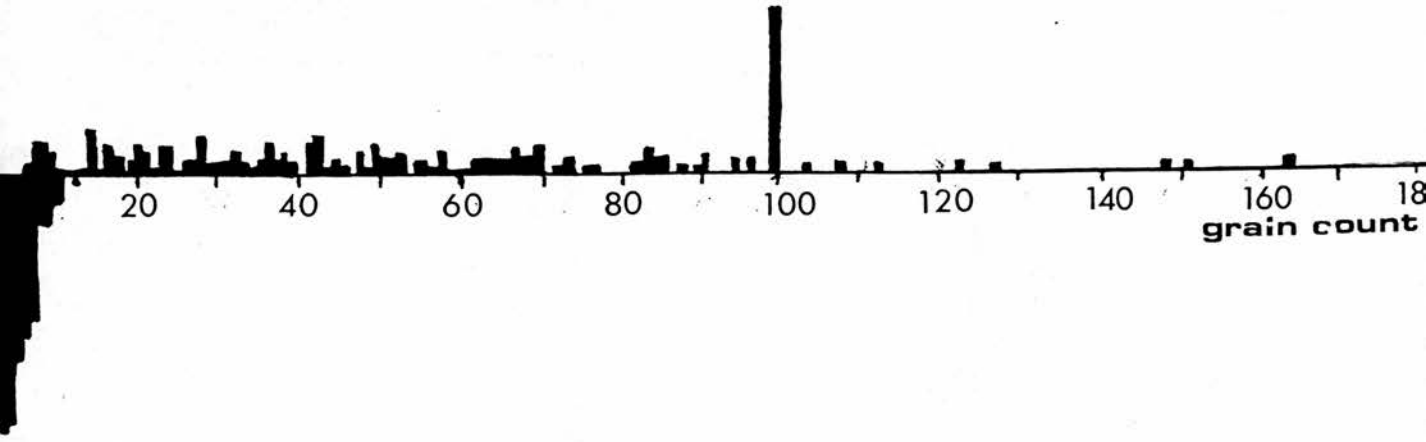
Figures 11a),11b) and 11c) represent the grain count analysis of three separate experiments using the transfer of tritiated uridine to measure the homotypic cooperation between derivatives of PR3.Experiments 11a) and 11b) correspond to the experiments 27/3 and 9/4 respectively and so cam be directly compared with the same experiments on cell lines in chapter 3 (figures 3:8c and 3:8d).


number of cells
Cell Line Ploidy(X) N1 N11 T NT T-NT T-NT/X %
Tg12Tx 5 100 100 23 4 19 3.8 94.6
5 100 100 16 2 14 2.8 97.4
5 200 200 19,5 3 16,5 3.3 96
Kd1a 5 100 100 9 2 7 1.4 80.1
5 100 100 3 0 3 0.6 83
5 100 100 19 3 16 3.2 90.5
5 300 300 10,3 1.7 8.6 1.7 84.6
Kd1a.6 5 100 100 13 1 12 2.4 90.4
5 100 100 16.5 4 12.5 2.5 91.1
5 100 100 12 3.5 8.5 1.7 82.1
5 300 300 13,8 2.8 11 2.2 87.9
Kd11Ba 5 100 100 10 5 5 1 63.6
5 100 100 9 3 6 1.2 83.7
5 200 200 9,5 4 5.5 1.1 73.7
grain coun
(a)


number of cells
Cell Line Ploidy(X) N1 N11 T NT T-NT T-NT/X %
Tg12 5 100 100 23 4 19 3.8 97.6
5 100 100 18,5 2 16,5 3.2 93.2
5 200 200 20,8 3 17,8 3.6 95.4
Tg12Tx 5 100 100 18.5 4 14.5 2.9 90
5 100 100 21 4 17 3.4 98.6
5 200 200 19,8 4 15,8 3.2 97.2
Kd1a 5 100 100 8 2 6 1.2 77.6
5 100 100 18,5 2.5 9 1.8 69
5 200 200 13,3 5.8 7.5 1.5 73.2
Kd1a.6 5 100 100 5 2 3 0.6 61.2
5 100 100 10 3 7 1.4 81.1
5 200 200 7,5 2.5 5 1 71.2
Kd..Ba 5 100 100 48.5 5 43.5 8.7 93.7
5 100 100 47 2.5 44.5 8.9 97.2
5 200 200 47,8 3.7 44.1 8.8 95.5
grain count
(a)

c)

Cell Line	Ploidy(X)	Ni	Nii	T	NT	T-NT	T-NT/X	%
Tg12	5	100	100	69	5	64	12.8	100
	5	100	100	33	5	28	5.6	98.4
	5	200	200	51	5	46	9.2	99.2



4.9.2. Cooperation of Cell Lines Selected by "Kiss of Death" from the Hybrid Line PR3

It would be expected that cell lines selected by "Kiss of Death" would have a reduced ability for gap junction-mediated communication.This is partially borne out by results reported in this section.

Tritiated Uridine analysis of the line Kd1a which was derived from PR3Tg12 through one "round" of the kiss of death procedure and of its clone Kd1a.6 show that both of these lines have considerably reduced metabolic cooperation,comparable to results obtained from the mec- parental line R5/3OA [Figure 3:8,Chapter 3].

Kd1a has T-NT/X values which vary from 0.6-3.2 with a mean of 1.6 and percentage cooperation estimates of 69-90.5%.Similarly Kd1a.6 has T-NT/X values of between 0.6 and 2.5 (mean 1.7) and a range of percentage cooperation estimates from 61.2-91.1.The T-NT/X value for R5/3OA in these experiments was between 1.08 and 1.5 and percentage cooperation estimate was between 63 and 83%.These figures are in close agreement with those of Kd1a and Kd1a.6 except that the range of values for the hybrid lines was larger with some cells apparently cooperating to a greater extent than cells of the R5/3OA line.

Results obtained in two separate experiments [Figures 4:11a and 4:11b] were quite similar for both lines Kd1a and Kd1a.6,although slightly higher T- NT/X values were obtained with the Kd1a.6 line in experiment 9/4 [Figure 4:11b].

The line Kd11Ba was derived from Kd1a via a further round of the "Kiss of Death" procedure [4.4].However,the results obtained from Uridine transfer analysis of this line were more difficult to interpret.Two experiments,apparently under identical conditions, [Figures 4:11a & 4:11b] produced completely different results.Figure 4:11a (27/3) shows the line Kd11Ba cooperating at least as well as the hybrid parent line PSA4 and to a greater extent than the hybrid line PR3 from which they were derived. T-NT/X values were 8.7- 8.9 (mean 8.8) and percentage cooperation estimates of 93.7- 97.2%,both of which are high values and indicate a mec+ line.

Experiment 9/4 [Figure 4:11b] however shows the opposite result with Kd11Ba

showing very little junctional communication with T-NT/X values of 1- 1.2 (mean1.1) and percentage cooperation estimates of 63.6-83.7%.These values are very low and indicate a mec- line.

This anomaly does not appear to be due to one of the two experiments (27/3 or 9/4) behaving uncharacteristically for some technical reason as results from all other lines set up in these two experiments are closely comparable and suggest reasonable reproducibility between experiments.

4.10. Summary

This chapter describes the further characterisation of one hybrid line,PR3,whose isolation and preliminary characterisation was described in Chapter 3.Cell lines were sucessfully isolated from the hybrid line PR3 described in Chapter 3,using selective medium alone.This was thought to be due to the process of chromosome loss characteristic of newly isolated hybrid lines [135].

A series of 18 cell lines (PR3Tg1-PR3Tg20) isolated from PR3 in 6- thioguanine are described.A survival frequency of approximately 1.5 per 10^3 cells was calculated (cf with [63]). for the HPRT gene,although this value varied by 35% either way depending on the concentration of thioguanine (10-30 µg/ml) used and the conditions in which the cells were plated.The relatively high frequency with which these lines were isolated argues that they have become 6-Tg resistant due to a reversion to the R5/3OA HPRT- phenotype by a mechanism of gene (chromosome) loss,rather than by a new mutation.This is supported by the results of plating efficiency experiments in HAT and Oua selective medium.All of the 6-Tg resistant lines tested were also,like the parent line R5/3OA,HAT sensitive (HPRT-),and were also all found to be resistant to Ouabain.

It was found that the range of cell survival was greater when cells were plated out into STO feeder conditioned medium than when cells were plated out onto feeders,it was therefore possible that the feeder cells afforded some non-specific protection against toxicity which allowed greater cell survival at higher (30µg/ml) concentrations of 6-Tg.Alternatively it could be that STO conditioned medium varies from batch to batch in its groth promoting ability and thus introduces more variability into the results than is obtained with STO feeder cell experiments.

At lower concentrations (10µg/ml) however the reverse was true and cell survival was greater in dishes without feeder cells which were fed with STO feeder conditioned medium.It would seem therefore that,although the overall reversion/mutation rate of cells plated in conditioned medium may be greater than in cells on feeders,but except at low concentrations of 6-Tg (10µg/ml) that this effect is masked by the ability of the STO feeder cells to afford some protection against toxicity.

Of the 18 permanent 6-Tg resistant cell lines isolated,all but one (PR3Tg1) were picked from dishes fed with 30µg/ml 6-TG.Ten of these lines (PR3Tg1-PR3Tg10) were isolated in STO feeder conditioned medium and the remaining eight on STO feeder cells.

Analysis of the karyotype of these cell lines has shown ,with the exception of PR3Tg10,that all of the lines karyotyped had a sub-hexaploid chromosome count,and that the majority had the two metacentric marker chromosomes present also in the parent line (PR3).Therefore all but one of these lines were apparently of hybrid origin.The remaining line PR3Tg10,had a modal chromosome number of 56 no metacentrics and a fibroblast morphology and was likely to be an STO cell contaminant.

Like the parent line (PR3) the 6-Tg resistant derivatives of this line displayed a range of chromosome counts centered around a sub-hexaploid mode.Those 6-Tg resistant lines selected in conditioned medium displayed a larger range of chromosome counts and a lower mean than those selected on STO feeder cells,giving additional support for the existance of different selective pressures operating on the two groups of variants.

One of these cell lines,PR3Tg12,was used in "Kiss of Death" experiments to isolate lines which were deficient in junctional communication.A Mec- line (Kd1a) was selected after only one round of the "Kiss of Death" procedure.This line was cloned and its clonal derivative,Kd1a.6 was also found to have very low levels of metabolic cooperation which were comparable with those of the Mec- parent line R5/3OA.

A line (Kd11Ba) selected from Kd1a through a further round of "Kiss of Death" selection gave ambiguous results in Uridine transfer experiments,with one experiment indicating a high level of cooperation and another indicating a low level of cooperation comparable to that of the Mec- lines R5/3OA,Kd1a and

Kd1a.6.No explanation for this anomalous result is immediately obvious,but it seems likely that it was caused by heterogeneity of the cell population so that a subpopulation of Mec+ cells overgrew the rest of the culture for some reason.This problem could be resolved by the cloning of the Kd11Ba line and by reexamining the cooperation properties of the clonal derivatives.

However,despite the anomalous result obtained with the Kd11Ba line,these results illustrate that the use of hybrid lines enables the selection of cooperation deficient lines by the use of thioguanine "kiss of Death" using fewer rounds of selection than has previously been necessary [1.7].The proposed hybrid origin of these lines was confirmed by karyotype analysis and the use of plating efficiency experiments in HAT,Ouabain and 6-Tg.

Analysis of the cooperation properties of the 6-Tg resistant hybrid (PR3Tg12) from which these lines were selected has shown that the level of metabolic cooperation in PR3Tg12 was comparable to and slightly greater than that found in the hybrid lines PR3 and PR3/4.This result confirms that the level of cooperation in these hybrid lines was substantially greater than that found in the Mec- parent line R5/3OA and indicate that selection in thioguanine had no observable effect on the metabolic cooperation properties of the hybrid line PR3.

However,a study of the differentiative properties of these cell variants has shown that although all of these lines (PR3Tg12,Kd1a,Kd1a.6 and Kd11Ba) behave like the parent line PSA4 and its hybrid derivatives PR3 and PR3/4 when induced to differentiate *in vitro* in embryoid bodies,neither the line PR3Tg12,or any of its derivatives could differentiate to any great extent *in vivo*.All of the mouse tumours derived from cells of the lines PR3Tg12,Kd1a.6 or Kd11Ba were formed predominantly of undifferentiated ec- like cells and resembled tumours derived previously from cells of the R5/3OA line.

This unexpected result demonstrates a difference between *in vivo* and *in vitro* differentiation which cannot be linked to the metabolic cooperation properties of these cell lines.Before making any further conclusion as to the mechanism of this observed difference it would,however,be prudent to first exclude the trivial explanation that these tumours were formed from a small population of fast-growing non-differentiating cells overgrowing the majority pluripotent cell type.This can be easily demonstrated by examining the *in vitro* differentiation

properties of cells isolated from such undifferentiated tumours.

A cell line (PR3Tg12TX) which was isolated from such a tumour (derived from cells of PR3Tg12) has already been karyotyped and was shown to have a similar range and mean of chromosome counts as the line PR3Tg12.The cooperation properties of this line have also been tested and have been shown to be identical to those of PR3Tg12.

CHAPTER 5

ISOLATION AND CHARACTERISATION OF CELL LINES WITH REDUCED DIFFERENTIATION ABILITY FROM THE HYBRID EC LINE PR3 AND ITS DERIVATIVES

As described in previous chapters [3.5.I,4.8.I],it has been observed that when the hybrid line PR3 and its derivatives are plated out onto gelatin in STO conditioned medium they display heterogeneity with respect to the extent of differentiation of colonies within the same dish.

This chapter describes the isolation of poorly differentiating cell lines from such cultures and investigates their properties with respect to karyotype,plating efficiency in HAT and Ouabain,extent of metabolic cooperation and *in vivo* and *in vitro* differentiation.

The lines described here therefore,complement those lines isolated by "Kiss of Death" selection and described in the previous chapter.An analysis of the two series of lines in conjunction should therefore increase the knowledge of the genetics of both gap junctional communication and differentiation in ec cell lines.

5.1. Isolation of Lines with Reduced Differentiation

Cell lines were isolated from dishes of cells plated out onto gelatin and fed with STO conditioned medium [2.3.3].Colonies were chosen for the absence of visible signs of differentiation.Colonies which maintained this morphology over the next 4 days [Figure 5:1] were then picked and transferred to cloning wells where they were grown up in STO conditioned medium until reaching confluence when permanent stocks could be made [2.5.4].

The illustrations in Figure 5:1 show an undifferentiated colony [5:1a] of the type which was picked in this selection procedure and an undifferentiated colony [5:1b] but associated with residual STO feeder cells.Colonies associated with STO feeder cells were avoided in selection because STO feeder cells inhibit the differentiation of colonies in culture so that possible pluripotency of such colonies would be masked by the presence of feeder cells.

Figure 5:1

SELECTION OF NON-DIFFERENTIATING COLONIES IN STO FEEDER CONDITIONED MEDIUM

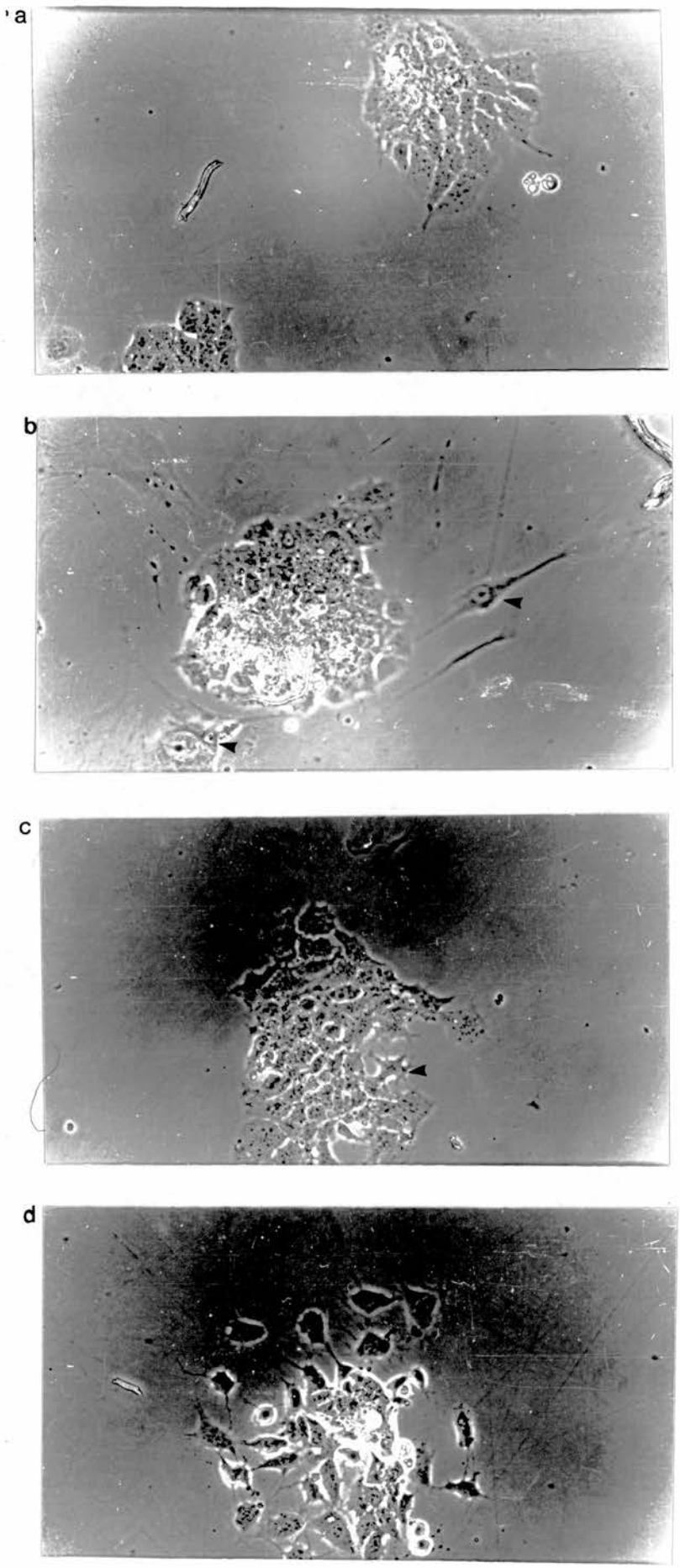
a) Undifferentiated colony growing in a dish of Kd11Ba cells plated out in STO feeder conditioned medium onto gelatin treated culture dishes.

Cell lines of this morphology were chosen as a basis for the isolation of the series of "D-Lines" which had impaired differentiation properties.

b) Undifferentiated colony from a dish of PR3Tg12 cells plated in STO conditioned medium on gelatin.This colony is associated with residual feeder cells (arrowed) which may be inhibiting these cells from differentiating.

c) Partially differentiated colony from a dish of Kd11Ba cells growing in STO conditioned medium on gelatin.A cell with differentiated morphology is arrowed.

d) Predominantly differentiated colony of PR3 cells growing on STO conditioned medium on gelatin.


a
b
c
d

The colonies shown in Figures 5:1c and 5:1d both show differentiation and were therefore not selected in this experiment.The colony in Figure 5:1c has only a few differentiated cells at one edge (arrow) whereas the colony shown in Figure 5:1d is almost completely differentiated.

Cell Variants (D-lines) were isolated as described above from PR3 and from two of its derivatives (PR3Tg12 and Kd11Ba).The variants isolated are summarised in Table 5:1.Six lines resulted from this series of selections,one (PR3.D1) from the parent hybrid line PR3,two (Kd11Ba.D1,Kd11Ba.D3) from the "Kiss of Death" selected hybrid Kd11Ba and three (PR3Tg12D1,PR3Tg12D2,PR3Tg12D3) from the 6- Tg resistant line PR3Tg12.A clonal line (PR3Tg12D1.1) was isolated from one of these lines (PR3Tg12D1) by dilution of the colony cells when they were transferred to cloning wells.

5.2. Characterisation of D-Lines

Cell lines isolated for reduced differentiation in conditioned medium,were karyotyped and their morphology observed under the light microscope.Their plating efficiency in HAT and Oua was also analysed and compared to the lines from which they were derived.

5.2.1. Morphology and Growth requirements of D-Lines

Like the hybrid lines from which they were isolated these variants all had an ec cell morphology with large nuclei containing several (4 or more) prominent nucleoli.All of these lines were routinely maintained in STO conditioned medium on gelatin as some differentiated colonies were always observed when these variants were plated onto gelatin in the absence of both conditioned medium and STO feeder cells.

5.2.2. Karyotyping of D-lines

The variants PR3Tg12D1,PR3Tg12D1.1,Kd11Ba.D1 and PR3D1 were karyotyped and the chromosomes of the metaphase spreads (approximately 20 per cell line) were counted and analysed for the presence of metacentrics.The results are illustrated in Figure 5:2 and show that,with the exception of the line

Table 5:1

SUMMARY OF CELL VARIANTS SELECTED FOR REDUCED DIFFERENTIATION IN STO CONDITIONED MEDIUM

Shows the parent line and method of isolation of each of the six lines isolated in STO conditioned medium to have reduced differentiation properties.

Line	Parent Line	How Selected	Remarks
PR3.D1	PR3	colony picked in Cond.Med.	
Tg12.D1	PR3Tg12	"	
Tg12.D2	PR3Tg12	"	Tg12.D1.1 is a cloned derivative
Tg12.D3	PR3Tg12	"	
Tg12.D1.1	Tg12.D1	cloning well dilution	clonal line
Kd11Ba.D1	Kd11Ba	colony picked in Cond.Med.	
Kd11Ba.D3	Kd11Ba	"	

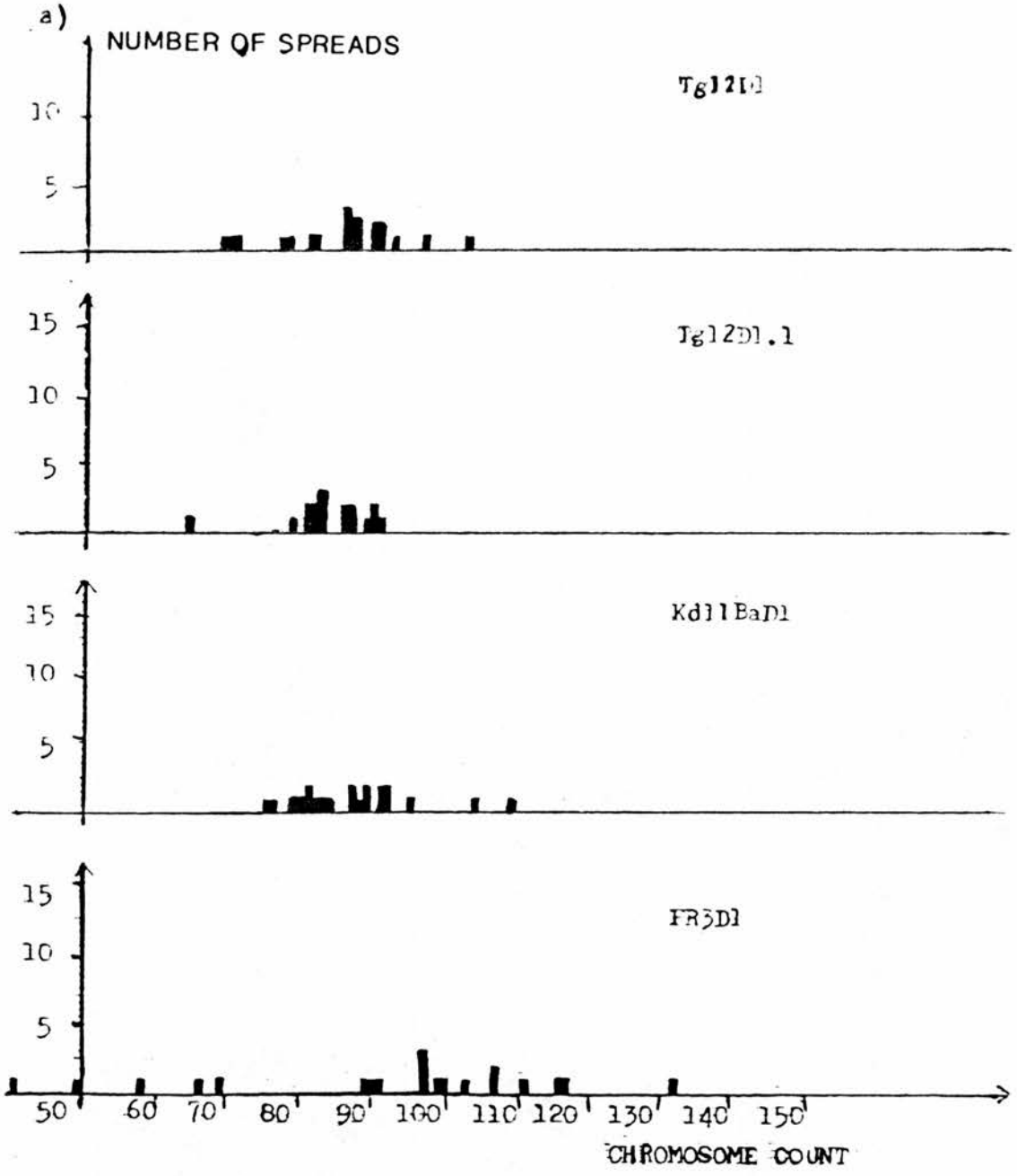
Figure 5:2

KARYOTPYE ANALYSIS OF HYBRID VARIANTS SELECTED FOR REDUCED DIFFERENTIATION

a) Histogram presentation of chromosome counts of Leishman's stained metaphase spreads of the hybrid variants PR3Tg12.D1,PR3Tg12.D1.1, Kd11Ba.D1 and PR3.D1.

b) Statistical analysis of the results shown in 5:2a showing that all variants have a subhexaploid karyotype resembling that of the original line PR3. With the exception of PR3Tg12D1 which has only one metacentric chromosome in some of its cells this aspect of the karyotype is also consistent with previous findings for hybrid variants.

a)



b)

Cell Line	Mode	Range	Mean	SD	Metacentrics
Tg12.D1	87	70-104	86	8.6	1-2
Tg12.D1.1	84	65-93	85.4	5.8	2
KdllBa.D1	-	76-110	89.1	8.9	2
FR3.D1	95	41-133	92.8	23.3	2

PR3Tg12D1 in which some cells appeared to have lost one of the metacentric markers,that all of these lines have retained the two metacentric chromosomes found in previous hybrids and derived originally from R5/3OA.

Considerable chromosome loss was observed in all variants karyotyped.The modal counts for the lines PR3Tg12D1 and PR3Tg12D1.1 (respectively 87 and 84) indicate a ploidy of approximately 4.5.The line KdIIBaD1 (with a mean chromosome count of 89) also has a ploidy of around 4.5.The karyotype of PR3D1 displayed a wide range of chromosome counts some of which were quite high and has a modal chromosome count of 98 indicating a ploidy of around 5.Typical metaphase spreads of these variants are illustrated in Figure 5:3.

5.2.3. Plating Efficiency of D- lines in HAT and Ouabain

The variants Kd11BaD1,PR3Tg12D1,PR3Tg12D2,PR3Tg12D3,PR3D1, were tested for their ability to grow in HAT and Oua as compared to the hybrid lines PR3Tg12,Kd11Ba and PR3 and the hybrid's parent lines R5/3OA and PSA4.The results of these plating efficiency experiments (2 experiments) are shown in Figure 5:4 and demonstrate that all of the D-lines conform to their origin with respect to resistance to HAT and Oua medium,so that with the exception of the PR3 derived line PR3D1 which like PR3 grows in all three types of media (HAT,Oua and HAT+Oua),all of these variants (Kd11BaD1,PR3Tg12D1,PR3Tg12D2,PR3Tg12D3) grow,like their respective parent lines,in medium containing Oua but not in medium containing HAT.

Therefore,the selection of these lines for reduced differentiation in STO conditioned medium did not affect either the Oua resistance or HPRT gene phenotype.With the exception of PR3D1 which was Oua^r and HPRT+,all of the D-lines isolated therefore had a phenotype like that of the line R5/3OA which is Oua^r and HPRT-.

5.3. Uridine Transfer Analysis of Metabolic Cooperation

The gap junction mediated transfer of tritiated Uridine between hybrid cells was assayed using autoradiography.Variants were analysed for homotypic metabolic cooperation (4 experiments) of 3H-Uridine between R5/3OA and hybrid line

Figure 5:3

PLATING EFFICIENCY OF DIFFERENTIATION DEFICIENT HYBRID VARIANTS IN HAT AND OUABAIN

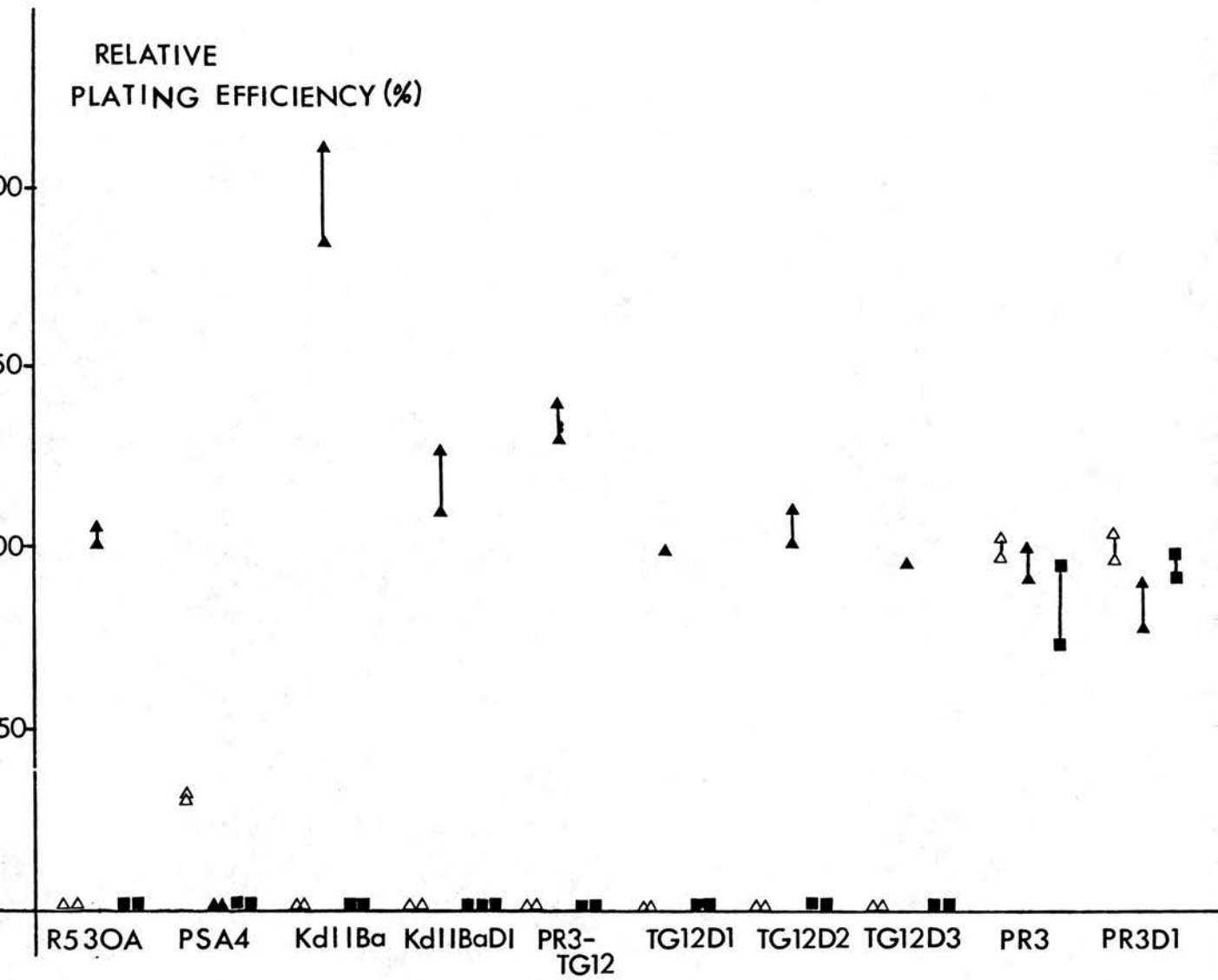
a) Graph plot of the result of plating efficiency experiments in medium containing HAT,Ouabain or both of these,showing that hybrid variants selected for reduced differentiation (D-Lines) have the same phenotype as the parent lines with respect to their HPRT gene and Ouabain resistance.

b) Statistical analysis of the results presented in 5:4a.

Key:

△ - HAT
▲ - Oua
■ - HAT + Oua

a)


RELATIVE
PLATING EFFICIENCY (%)
00
50
00
50
R530A
PSA4
Kd11Ba
Kd11BaD1
PR3-
TG12
TG12D1
TG12D2
TG12D3
PR3
PR3D1

b)

Cell Line	HAT	Oua	HAT+Oua	
PSA4	31.4	0.35	0.1	mean
	0.55	0.07	0.07	SD
R5/30A	0	107.6	0	mean
	0	1.41	0	SD
KdllBa	0.15	198.7	0	mean
	0.21	18.9	0	SD
KdllBa.Dl	0	120	0	mean
	0	13.5	0	SD
Tgl2	0	136.6	0	mean
	0	6.6	0	SD
Tgl2.Dl	0	99.9	0	mean
	0	-	0	SD
Tgl2.D2	0	107	0	mean
	0	6.6	0	SD
Tgl2.D3	0	108.8	0	mean
	0	-	0	SD
PR3	99.9	95.7	84.7	mean
	2.6	4.4	10.9	SD
PR3.Dl	100.2	83.8	82.8	mean
	3.9	6.3	1.6	SD

variants.

5.3.1. Analysis of Homotypic transfer of 3H-Uridine between Cells of the Hybrid Derived D-lines

The analysis of uridine transfer in all four experiments [Figure 5:5] shows that the line PR3Tg12D1 can cooperate quite extensively with an T-NT/X value of 4-5 and a percentage cooperation estimate of between 91 and 95.This ability to cooperate is shared by the lines PR3Tg12D3 (1 experiment) with an T-NT/X value of 3.4 and percentage cooperation estimate of 89 and also by PR3Tg12D2 (2 experiments) with T-NT/X values of 3.4 and 7.9 and percentage cooperation estimates of 95-99%.The line PR3D1 (1 experiment) can also cooperate well with an T-NT/X value of 7.1 and percentage cooperation estimate of 98%.These results are summarised in Table 5:4.

These values,although somewhat reduced in comparison to the level of cooperation found in PSA4 [3.4.1] are comparable to cooperation levels of the mec+ hybrid PR3 and its mec+ derivatives.

These results are in contrast to those of the lines PR3Tg12D1.1,Kd11BaD1,and Kd11BaD3 all of which are shown to have low levels of cooperation.The clonal line (PR3Tg12D1.1) derived from PR3Tg12D1 is shown to have a different,lower cooperation ability (3 experiments) with T-NT/X values of 0.8-2.3 and a percentage cooperation estimate of between 68 and 83.The lines Kd11BaD1 (2 experiments) and Kd11BaD3 (2 experiments) are both shown to be of reduced cooperation ability with T-NT/X values of 2 and 1.3-3 respectively with mean percentage cooperation estimates of 84% and 90% respectively.These results indicate cooperation levels comparable to those of the mec- line R5/3OA [3.4.1].

5.4. Differentiation of hybrid derived D-Lines

The cell lines PR3Tg12D1,PR3Tg12D1.1,PR3Tg12D2,PR3Tg12D3,PR3D1 and Kd11BaD1 were analysed for their ability to differentiate both *In Vivo* and *In Vitro*.

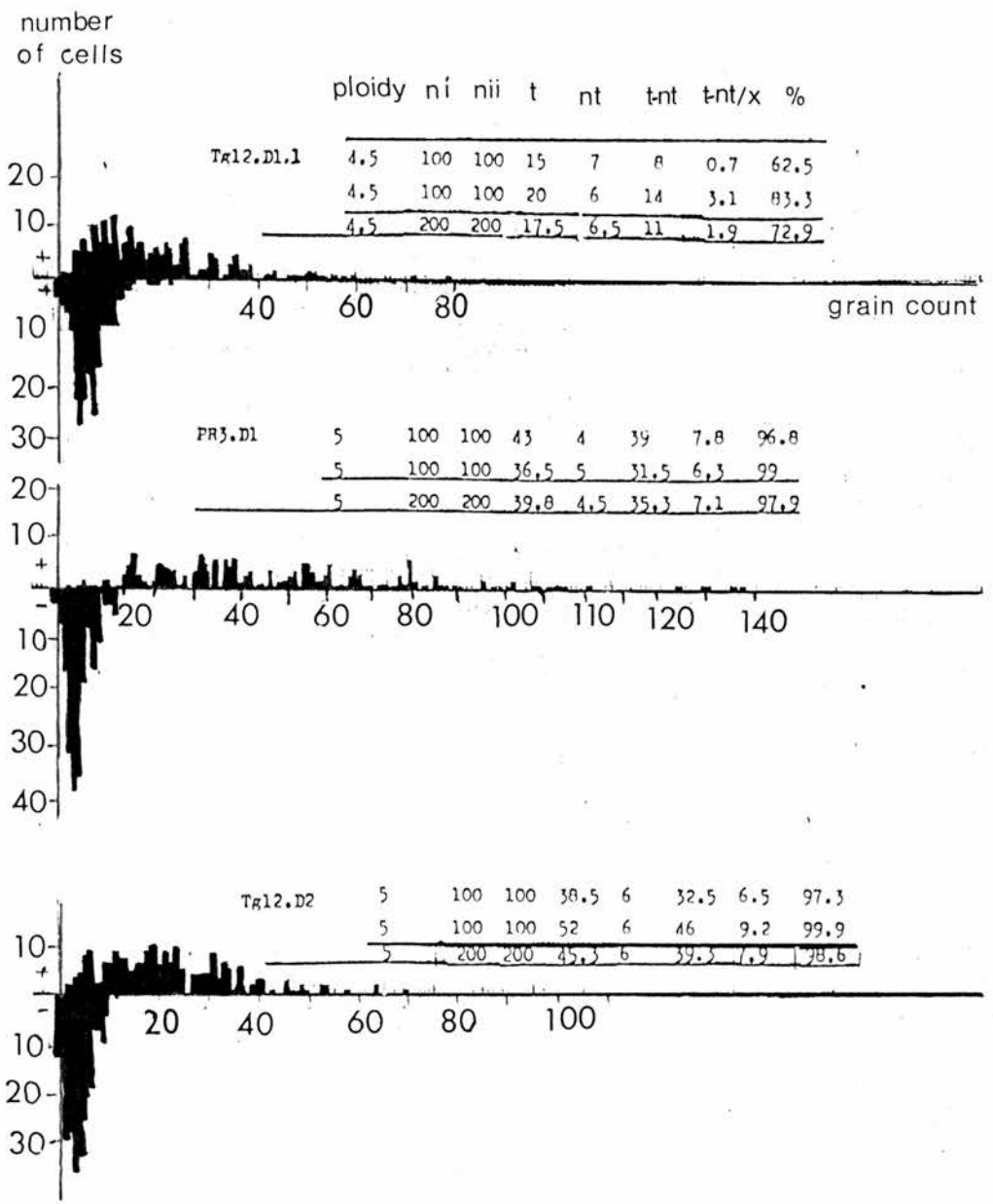
Figure 5:4

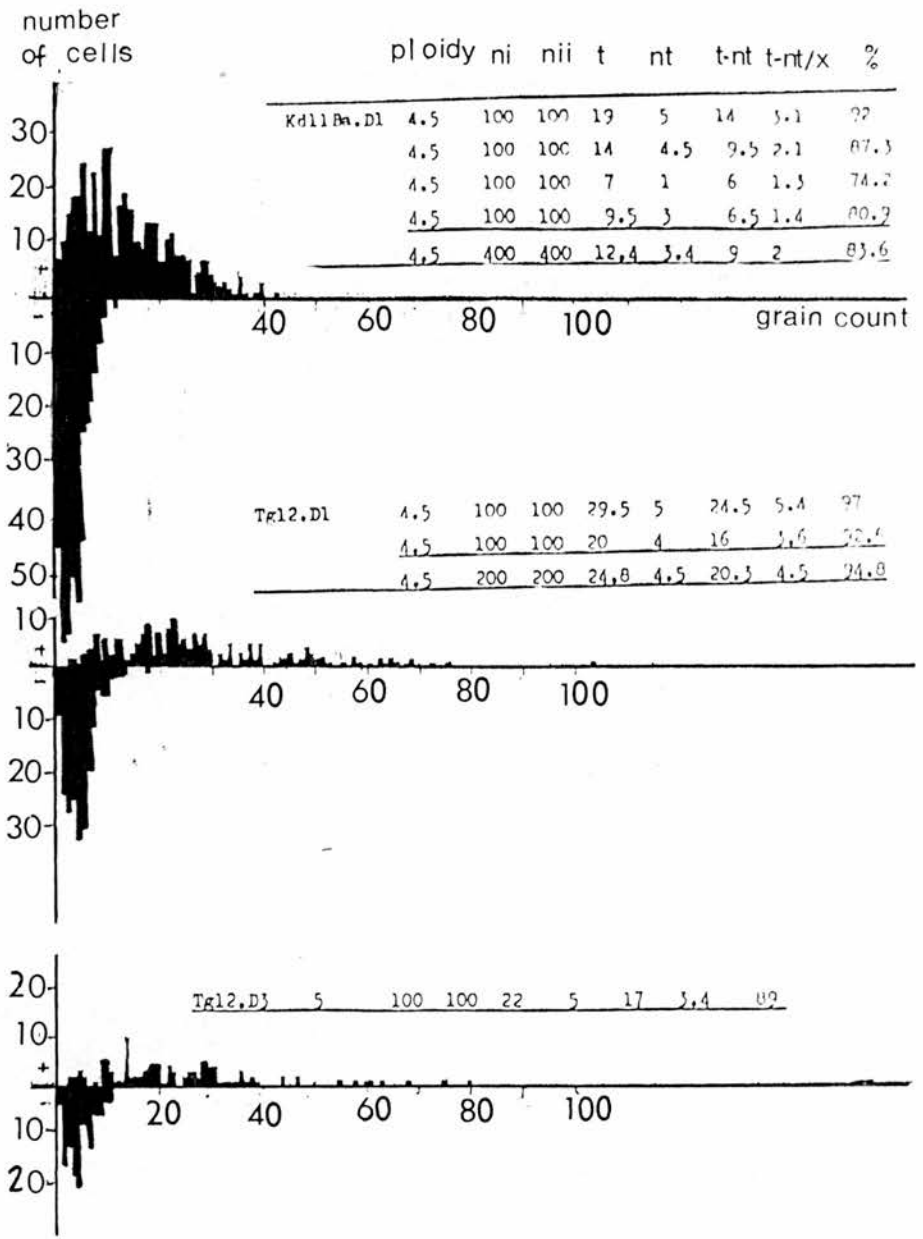
TRITIATED URIDINE TRANSFER ANALYSIS OF THE METABOLIC COOPERATION OF HYBRID VARIANT LINES WITH REDUCED DIFFERENTIATION PROPERTIES

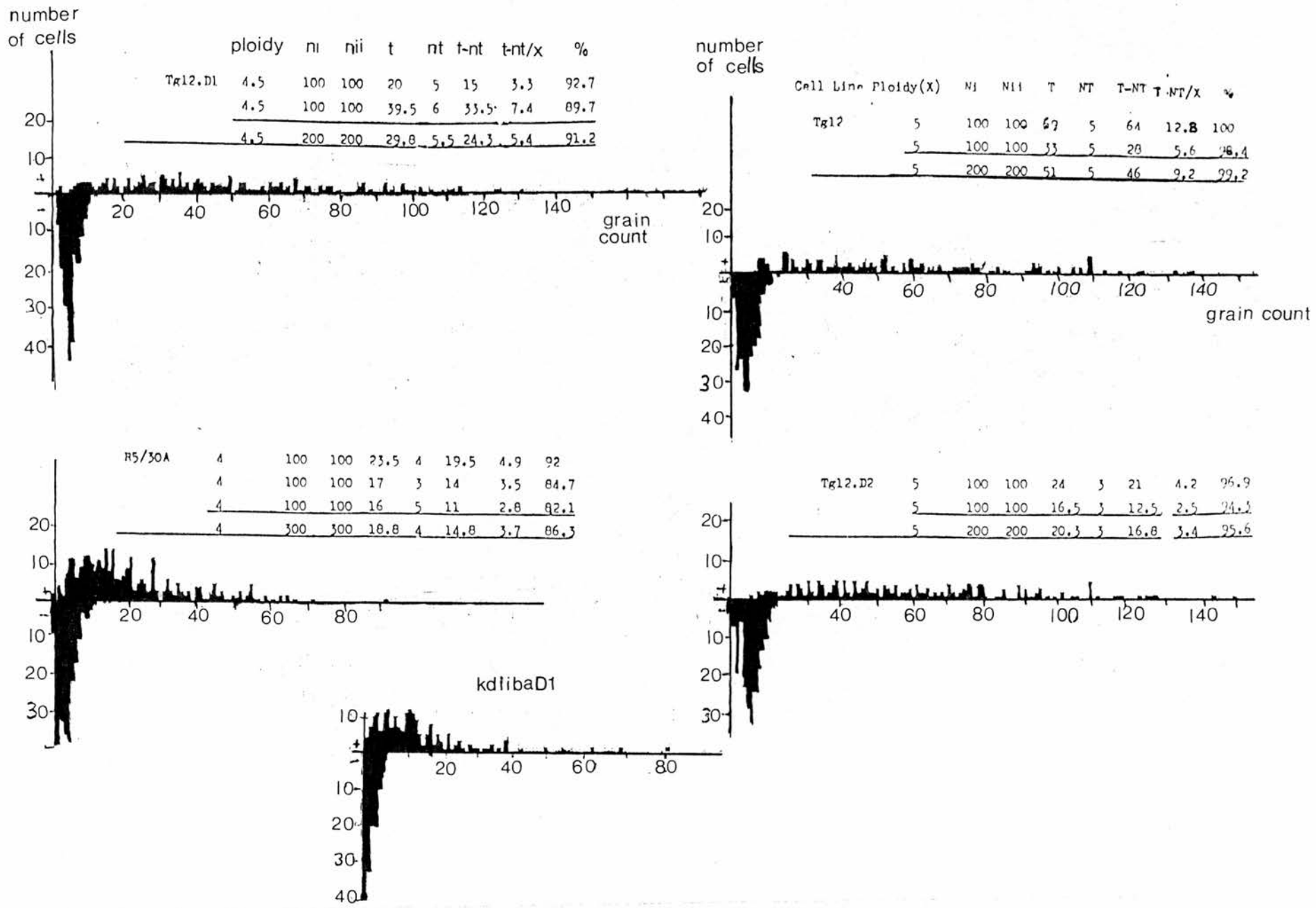
Homotypic analysis of the transfer of radioactive nucleotides between hybrid variant lines in contact with each other.

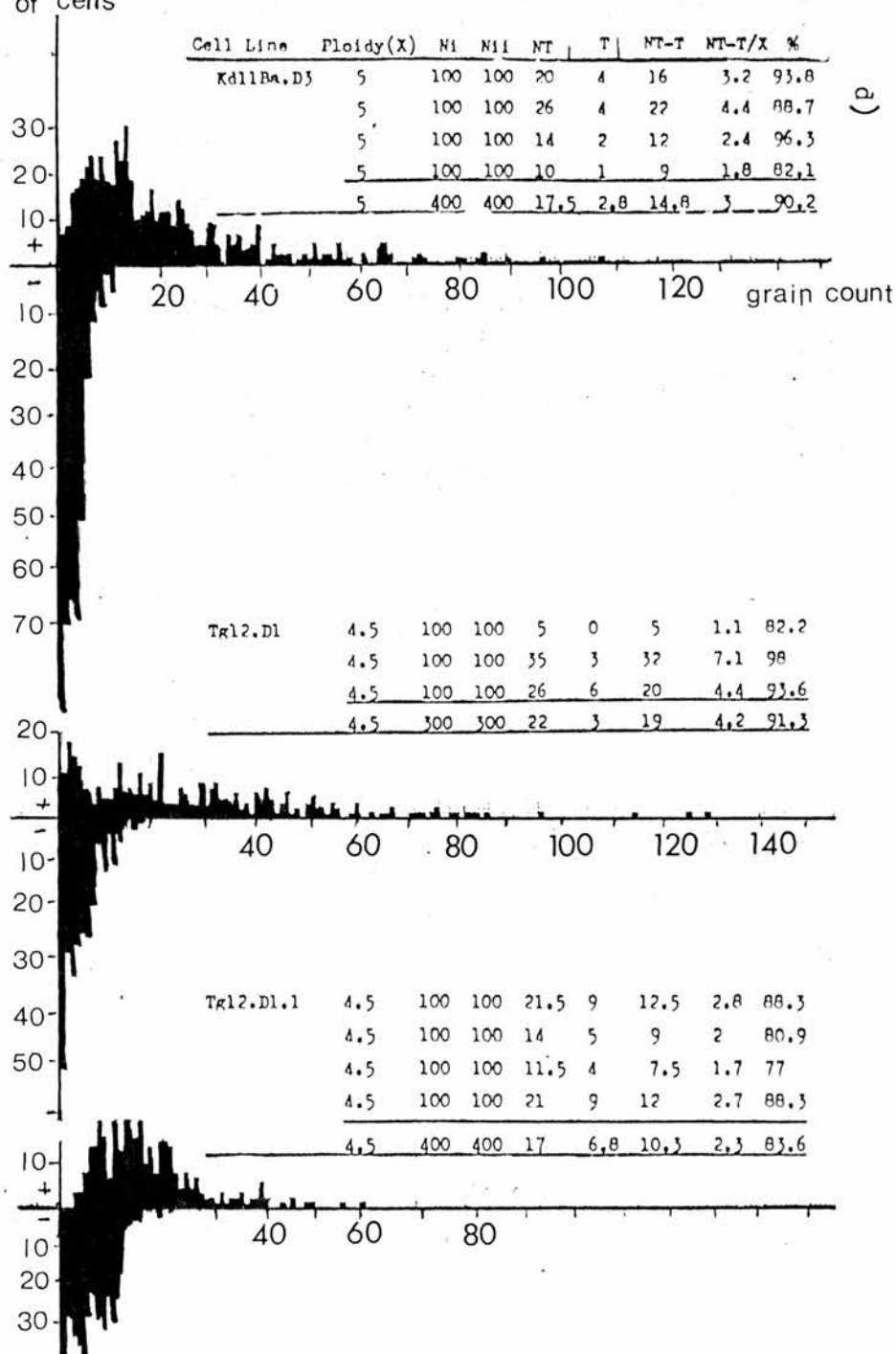
a),b),c) and d) represent four separate experiments.c) is equivalent to the experiment 27/3 and d) to experiment 9/4,so the results in these two can be directly compared to the corresponding experiments in chapter 3 (3:8c and 3:8d) and in chapter 4 (4:11a and 4:11b).

Results show that the variants PR3Tg12D1.1,Kd11BaD3 and Kd11BaD1 all have significantly reduced cooperation abilities comparable to those of the mec- line R5 3OA,while the remainder have an intermediate or mec+ phenotype.

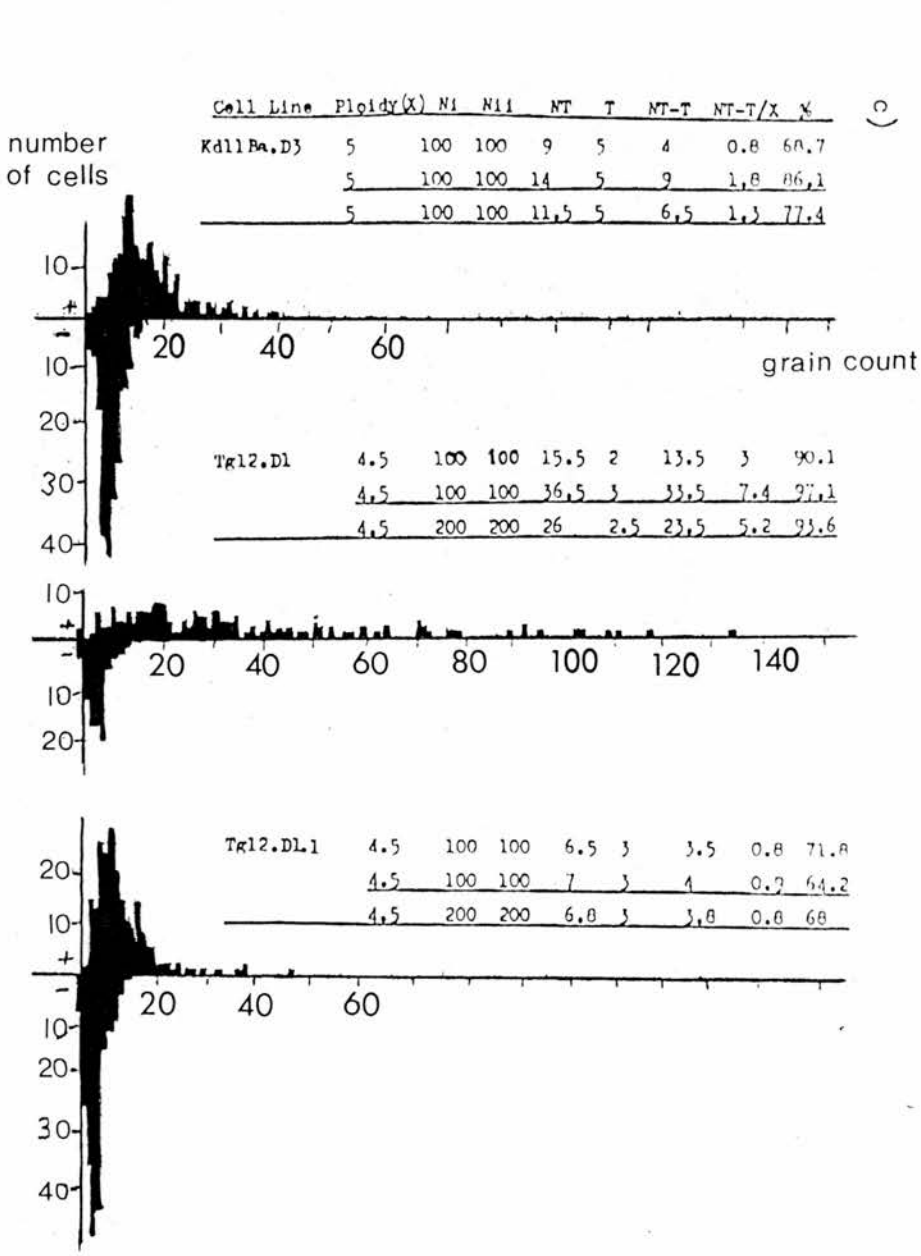

number
of cells
ploidy ni nii t nt t-nt t-nt/x %
Tg12.D1.1 4.5 100 100 15 7 8 0.7 62.5
4.5 100 100 20 6 14 3.1 83.3
4.5 200 200 17.5 6.5 11 1.9 72.9
PR3.D1 5 100 100 43 4 39 7.8 96.8
5 100 100 36,5 5 31.5 6,3 99
5 200 200 39.8 4.5 35,3 7.1 97.2
Tg12.D2 5 100 100 38.5 6 32.5 6.5 97.3
5 100 100 52 6 46 9.2 99.9
5 200 200 45.3 6 39.3 7.9 98.6
grain count


number
of cells
ploidy ni nii t nt t-nt t-nt/x %
Kd11Ba.D1 4.5 100 100 19 5 14 3.1 77
4.5 100 100 14 4.5 9.5 2.1 87.3
4.5 100 100 7 1 6 1.3 74.2
4.5 100 100 9.5 3 6.5 1.4 80.2
4.5 400 400 12,4 3.4 9 2 83.6
Tg12.D1 4.5 100 100 29.5 5 24.5 5.4 97
4.5 100 100 20 4 16 3.6 90.4
4.5 200 200 24,8 4.5 20.3 4.5 94.8
Tg12.D3 5 100 100 22 5 17 3.4 89
grain count


number of cells
ploidy ni nii t nt t-nt t-nt/x %
Tg12.D1 4.5 100 100 20 5 15 3.3 92.7
4.5 100 100 39.5 6 33.5 7.4 89.7
4.5 200 200 29.8 5.5 24.3 5.4 91.2
grain count
number of cells
Cell Line Ploidy(X) Ni Nii T NT T-NT T-NT/X %
Tg12 5 100 100 69 5 64 12.8 100
5 100 100 33 5 28 5.6 98.4
5 200 200 51 5 46 9.2 99.2
grain count
R5/30A 4 100 100 23.5 4 19.5 4.9 92
4 100 100 17 3 14 3.5 84.7
4 100 100 16 5 11 2.8 82.1
4 300 300 18.8 4 14.8 3.7 86.3
Tg12.D2 5 100 100 24 3 21 4.2 96.9
5 100 100 16.5 3 12.5 2.5 94.3
5 200 200 20.3 3 16.8 3.4 95.6
kdlibaD1



Cell Line	Ploidy(X)	Ni	Nii	NT	T	NT-T	NT-T/X	%
Kd11Ba.D3	5	100	100	20	4	16	3.2	93.8
	5	100	100	26	4	22	4.4	88.7
	5	100	100	14	2	12	2.4	96.3
	5	100	100	10	1	9	1.8	82.1
	5	400	400	17.5	2.8	14.8	3	90.2
Tg12.D1	4.5	100	100	5	0	5	1.1	82.2
	4.5	100	100	35	3	32	7.1	98
	4.5	100	100	26	6	20	4.4	93.6
	4.5	300	300	22	3	19	4.2	91.3
Tg12.D1.1	4.5	100	100	21.5	9	12.5	2.8	88.3
	4.5	100	100	14	5	9	2	80.9
	4.5	100	100	11.5	4	7.5	1.7	77
	4.5	100	100	21	9	12	2.7	88.3
	4.5	400	400	17	6.8	10.3	2.3	83.6



Cell Line	Ploidy(X)	Ni	Nii	NT	T	NT-T	NT-T/X	%
Kd11Ba.D3	5	100	100	9	5	4	0.8	60.7
	5	100	100	14	5	9	1.8	86.1
	5	100	100	11.5	5	6.5	1.3	77.4
Tg12.D1	4.5	100	100	15.5	2	13.5	3	90.1
	4.5	100	100	36.5	3	33.5	7.4	97.1
	4.5	200	200	26	2.5	23.5	5.2	93.6
Tg12.D1.1	4.5	100	100	6.5	3	3.5	0.8	71.8
	4.5	100	100	7	3	4	0.9	64.2
	4.5	200	200	6.8	3	3.8	0.8	68

5.4.1. *In Vitro* Differentiation of D-Lines

Like the lines from which they were derived,colonies of the variants PR3D1,Kd11BaD1,PR3Tg12D1,Tg12D1.1,PR3Tg12D2 and PR3Tg12D3 all displayed some heterogeneity in the extent of their differentiation when plated onto gelatin in STO conditioned medium.

However,embryoid body experiments using these cells revealed that,in comparison to the extent of differentiation in embryoid bodies (EB) formed from PR3 cells in the same experiments,the lines PR3Tg12D1,PR3Tg12D1.1,PR3Tg12D2 and PR3Tg12D3 all had a substantially reduced ability to differentiate.EBs from the line Kd11baD1 were of an intermediate extent of differentiation,while PR3D1 embryoid bodies were almost as differentiated as the parent line.This latter result may however be due to the small sample size of EBs available for the assessment of PR3D1 differentiation.All of these results are tabulated and shown in Pie-Chart form in Figure 5:5.

When these cell lines were compared to the lines from which they were directly derived it was found that Kd11BaD1 had virtually the same pattern and extent of differentiation as Kd11Ba,EBs from PR3D1 were slightly reduced in the extent of their differentiation,while the lines derived from PR3Tg12 all showed a substantial reduction in the extent of their differentiation when compared PR3Tg12 [Figure 4:9],the majority of EBs formed being completely undifferentiated.Some of these undifferentiated EBs were associated with an outer rind of necrotic cells (Undifferentiated category II) which possibly represented abortive endoderm differentiation.This feature has also been observed in R5/3OA [3.5.2].A small proportion of EBs also had identifiable endoderm differentiation and very occasionally the beginnings of cavity formation were also observed [Figure 5:5].

5.4.2. *In Vivo* Differentiation of D-Lines

Tumours were found in mice injected with the lines PR3Tg12D1 ,PR3Tg12D3 and PR3Tg12 in Tumour Experiment 2 [Table 5:2,and see also Tables 4:4 and 4:5 for report of PR3Tg12 tumours).

It was found that all of the tumours derived in this experiment

Figure 5:5

ANALYSIS OF *IN VITRO* DIFFERENTIATION IN EMBRYOID BODIES OF PR3 DERIVED VARIANTS SELECTED FOR REDUCED DIFFERENTIATION

a) Total counts of embryoid bodies falling into each of the five categories listed.(one experiment)

b) Pie-chart representation of the results given in 5:8a.Two categories of undifferentiated are defined

1) Completely undifferentiated
11) Embryoid bodies associated with an external ring of necrotic and possibly differentiating cells which cannot be positively identified as endoderm and so do not fit into categories 2 or 3 indicating varying degrees of endoderm differentiation.

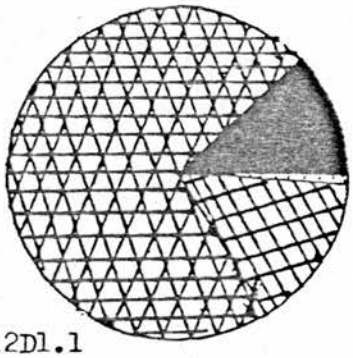
Key:

- 1
- 11
- VE,PE (+)
- VE,PE (++)
- VE,PE,cavity formation (+)
- VE,PE,cavity formation (++)

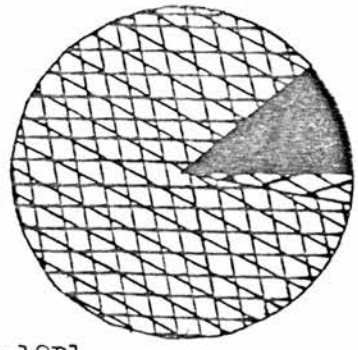
a)

Cell Line	Undifferentiated 1	11	VE PE +	VE PE ++	VE PE Cavity +	VE PE Cavity ++
PR3Tg12D1.1	57	323	69	-	3	-
PR3Tg12D1	94	708	19	-	-	-
PR3Tg12D3	41	266	96	-	1	-
PR3Tg12D2	76	405	48	-	2	-
Kd11BaD3	35	-	252	97	34	2
PR3D1	1	-	3	18	5	1

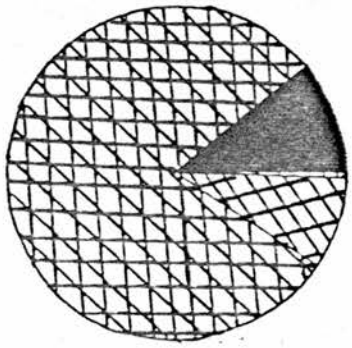
b)



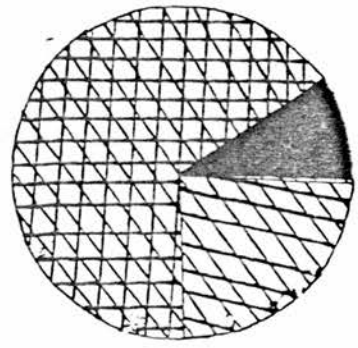
PR3Tg12D1.1



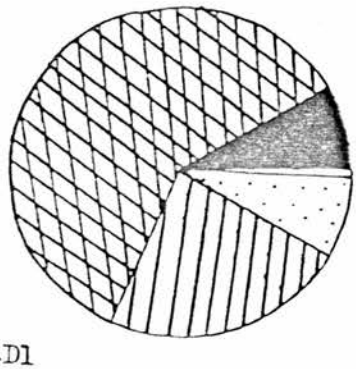
PR3Tg12D1



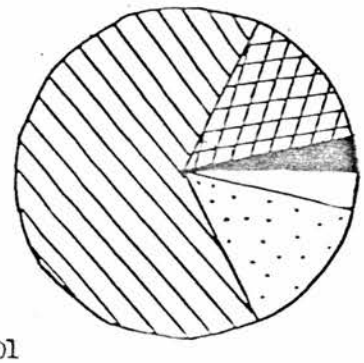
PR3Tg12D2



PR3Tg12D3



Kd11BaD1



PR3D1

Table 5:2

TUMOURS DERIVED FROM THE HYBRID VARIANTS PR3TG12D1 AND PR3TG12D3

Tumours were obtained from cells of the PR3Tg12 hybrid derived lines Tg12D1 and Tg12D3 and like the parent line were found to form tumours in mice which were formed predominantly of undifferentiated ec cells.

The tumours reported in this table correspond to tumour experiment 2 as described in chapter 4 (Table 4:4).

The following results were obtained from syngenic mice injected with cells of one of these lines,

PR3Tg12D1	2/4	tumours formed
PR3Tg12D3	2/3	tumours formed

animals remaining after 9 months were killed and autopsied with no further tumours being found.

Cell Line	Passage number	Sex of animal	Days to form tumour	Remarks
PR3Tg12D1	3	M	42	1.5 cm tumour n.o.a.b.
	3	M	62	2 cm tumour with a good blood supply n.o.a.b
	3	F	N A	no tumour
	3	F	N A	no tumour
PR3Tg12D3	2	F	25	2 cm tumour with good blood supply uterus slightly enlarged
	2	F	51	1.5 cm tumour n.o.a.b.
	2	M	N A	no tumour

notes:

N A - not applicable
n.o.a.b. - no other abnormalities found
M - male mouse
F - female mouse

Table 5:3

ANALYSIS OF THE *IN VITRO* DIFFERENTIATION IN TERATOCARCINOMAS FORMED IN MICE BY THE INJECTION OF CELLS OF THE VARIANTS PR3TG12D1 AND PR3TG12D3

Shows the results of scoring mouse tumours derived from PR3Tg12D1 or PR3Tg12D3 for the presence or absence of eleven categories of differentiated tissue and also for the presence or absence of undifferentiated ec cells.The table shows that all tumours derived from these two cell lines were formed predominantly of undifferentiated ec lines.

1 - Cartilage
2 - Bone
3 - Haematopoiesis
4 - Putative Trophectoderm
5 - Neural tube formation
6 - Glandular epithelium
7 - Keratinising epithelium
8 - Ciliated epithelium
9 - Smooth muscle
10 - Striated muscle
11 - Columnar epithelium
(12 - Embryonal carcinoma cells)

notes:

i) plus signs in brackets - (+) - indicate that this category is present in some,but not all tumours scored.

ii) Double plus signs - ++ - indicate that the cell type (ec) is the predominant tissue in that tumour.

iii) Plus signs - + - indicate presence and minus signs - indicate absence of a tissue type.

Cell Line	1	2	3	4	5	6	7	8	9	10	11	(12)
PR3Tg12D1	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
	+	-	-	-	-	-	-	-	+	+	-	++
PR3Tg12D3	+	-	-	-	+	-	-	-	-	+	-	++
	-	-	-	-	-	-	-	-	+	+	-	++
	(+)	-	-	-	(+)	-	-	-	(+)	+	-	++

Table 5:4

A SUMMARY OF THE PHENOTYPIC PROPERTIES OF HYBRID CELL LINES SELECTED IN STO CONDITIONED MEDIUM

Summarises the results of Uridine transfer experiments (metabolic cooperation) tumour formation and embryoid body differentiation,and plating efficiency tests (Ouabain resistance and presence or absence or functional HPRT enzyme).

notes:

i) Difb - differentiation in embryoid bodies (*in vitro* differentiation)

ii) Dift - differentiation in mouse tumours (*in vivo* differentiation)

iii) $T-NT_X$ - median of 'touching' counts minus median of 'non-touching' counts adjusted for ploidy (X).(Measurement of the extent of metabolic cooperation).

iv) %age- percentage of contacting cells which have a high degree of cooperation as measured by computor analysis.

v) nd - indicates that the experiment was not done.

l Line		Ploidy	T-NT/X	%age		Phenotype in vitro	Phenotype in vivo		
D1	5a	5	7.1	97.9	mec^+	$Difb^+$	nd	$HPRT^+$	Oua^r
1BaD3	5c	5	3	90.2	mec^-	nd	nd	nd	nd
	5d	5	1.3	77.4					
1BaD1	5a	4.5	2	83.6	mec^-	$Difb^+$	nd	$Hprt^-$	Oua^s
	5b								
2D1	5a	4.5	4.5	94.8	mec^+	$Difb^-$	$Dift^-$	$Hprt^-$	Oua^r
	5b	4.5	5.4	91.2					
	5c	4.5	5.2	93.6					
	5d	4.5	4.2	91.3					
2D1.1	5a	4.5	1.9	72.9	mec^-	$Difb^-$	nd	nd	n
	5c	4.5	0.8	68					
	5d	4.5	2.3	83.6					
2D2	5a	5	7.9	98.6	mec^+	$Difb^-$	nd	$Hprt^-$	Ou
	5b	5	3.4	95.6					
2D3	5a	5	3.4	89	mec^+	$Difb^-$	$Dift^-$	$Hprt^-$	Ou

were predominantly of undifferentiated ec cell type.The sections taken from each tumour were analysed [Table 5:3] and scored for the presence or absence of a variety of differentiated tissue types.It was found that tumours derived from the injection of PR3Tg12D1 cells were all predominantly ec cells but also contained differentiated areas containing cartilage,Smooth muscle and striated muscle.

Similarly tumours of PR3Tg12D3 cells were composed mainly of ec cells but also had small areas of differentiated tissues.Both tumours derived from PR3Tg12D3 had smooth muscle and additionally one had striated muscle while the other had areas of cartilage and neural tube formation.

5.5. Summary

This chapter describes a novel method for the isolation of cell variants with reduced differentiative capacity.The method utilises the observation that STO conditioned medium inhibition of ec cell differentiation is incomplete in the absence of feeder cells and that in cultures of the hybrid line PR3 and its variants there is a heterogeneity with respect to the extent of colony differentiation.Because of the ease with which variants are selected from these hybrids it seemed possible that this heterogeneity represented genetic differences between the cells of the colonies which could be selected to produce new variant lines.

Of six lines and one subclone isolated using this method,four variants (PR3Tg12D1 and its subclone PR3Tg12D1.1,PR3Tg12D2,PR3Tg12D3) were found to have substantially reduced differentiation properties *in vitro* as measured by embryoid body formation,two (KdIIBaD1 and PR3D1) were found to differentiate in embryoid bodies almost as well as the lines from which they were derived and one (KdIIBaD3) has not yet been investigated in this respect.

Difficulty was experienced in obtaining tumours from these variants but two lines (PR3Tg12D1 and PR3Tg12D3) were shown to produce tumours which were poorly differentiating and predominantly of ec cell type.However the 6-thioguanine resistant derivative of PR3 (PR3Tg12) from which both of these variants were selected also gives poorly differentiating tumours on injection into syngenic mice.

Morphologically all of these variants are characteristically of ec cell type with large nuclei,little cytoplasm and several prominent nucleoli.Karyotyping has revealed considerable chromosome loss in all of the lines (PR3Tg12D1,PR3Tg12D1.1,KdIIBaD1 and PR3DI) whose karyotype was analysed,with a ploidy of between 4.5 and 4 compared to the sub-hexaploidy of the original hybrid (PR3).

Plating efficiency experiments have shown that (with respect to the HPRT gene and Ouabain resistance) these lines have the same phenotype as those variants from which they were isolated so that PR3D1 will grow in both HAT and Ouabain containing medium,while the remaining lines (KdIIBaD1,PR3Tg12D1,PR3Tg12D2,PR3Tg12D3) will grow in Ouabain but not in HAT.

Uridine transfer analysis of the metabolic coopeation properties of variants discussed in this chapter indicate no relationship between their potency and their cooperation abilities.The line PR3D1 is mec+ and diff+,the line KdIIBaD1 is mec- and diff+ while the lines PR3Tg12D3,PR3Tg12D2 and PR3Tg12D1 are all mec+ and Diff-.The subclone of PR3Tg12D1 (PR3Tg12D1.1) was found to resemble the R5/3OA line in that it was both mec- and Diff-.

CHAPTER 6

DISCUSSION OF THE PROPERTIES OF HYBRID LINES CONSTRUCTED FROM THE EC LINES PSA4 AND R5/3OA AND SEGREGANTS ISOLATED FROM ONE OF THESE HYBRIDS

In the preceding chapters I have described the isolation and characterisation of a variety of hybrid cell lines using PEG mediated somatic cell fusion and the analysis of these lines using somatic cell genetics techniques.In this chapter I shall discuss the significance of these results in the furtherance of knowledge of cellular differentiation and communication and their value in context with relevant research already carried out by other workers and reviewed in the introduction to this thesis [Chapter 1].

This thesis has described the adaptation and refinement of existing methods for the chemical fusion of eukaryotic cells to form a hybrid containing a composite genome derived from more than one genetically different source.The technique of somatic cell fusion has already been shown [1.6..] to be a powerful tool in the elucidation of the genetic background of a variety of mechanisms and is likely to be especially important in the study of the genetics of differentiation and its possible role in tumour formation.

The use of chemical fusogens to create hybrid cell lines poses particular problems when embryonal carcinoma cells are used and in the past it has proved difficult to obtain such lines [1.6].The development of STO conditioned medium [3.1.3] and its use in the place of STO feeder cells in the selection of hybrid lines has allowed the use of a HAT and Ouabain selective medium procedure [1.6.] even when feeder dependent and cooperation positive lines such as PSA4 are used.The original selection procedure of Rosenstraus et al was found not to be effective because of the "Ouabain rescue" effect of the Ouabain resistant STO feeder cells which cooperated with the Mec+ PSA4 cells enabling them to survive in Ouabain.This invalidated the selection system which is dependant on the combination of HAT sensitive (R5/3OA) cells with Ouabain sensitive (PSA4) cells allowing the survival only of hybrid cells in the composite medium.

It was necessary to arrive at a compromise density at which to plate out the cocultures of cells.If the density was too great there was a danger of

Table 6:1

A SUMMARY OF THE PROPERTIES OF CELL LINES ISOLATED FROM FUSOGEN TREATED CO-CULTURES OF PSA4 AND R5 30A AND OF VARIANTS SELECTED FROM ONE OF THESE LINES (PR3) BY "KISS OF DEATH" AND "CONDITIONED MEDIUM" SELECTION PROCEDURES

Summarises the results of characterising cell lines whose isolation is reported in chapters 3,4 and 5 of this thesis.

Key:

nd - not done
diff - differentiation
mec - metabolic cooperation
s - sensitive (to Ouabain)
r - resistant (to Ouabain)

notes:

i) numbers marked (') indicate that mean chromosome count is quoted rather than the mode.

Cell Line	Cooperation (T-NT/X range)	Differentiation in vitro	Differentiation in vivo	HPRT	Oua	Karyotype mode	Karyotype metacentric
PSA4	mec+ 6-10.5	diff+	diff+	+	s	40	0
R5/30A	mec- 1.1-3.7	diff-	diff-	-	r	68	2
PR3	mec+ 4.2-9.6	diff+	diff+	+	r	102	2
PR3/4	mec+ 2.1-8.1	diff+	nd	+	r	100	1-2
PR5	mec+ 3.3-16	diff+	diff+	+	r	38	0
PR3Tg12	mec+ 3.6-9.2	diff+	diff-	-	r	116	2
PR3Tg12TX	mec+ 3.3-3.6	nd	nd	nd	nd	111	1-2
Kd1a	mec- 1.5-1.7	nd	nd	-	r	112	2
Kd1a.6	mec- 1-2.2	diff+	diff-	-	r	85.8'	1
Kd11Ba	mec+ 1.1-8.8	diff+	diff-	-	r	108	2
PR3D1	mec+ 7.1	diff+	nd	+	r	98	2
PR3Tg12D1	mec+ 4.2-5.4	diff-	diff-	-	r	87	1-2
PR3Tg12D1.1	mec- 0.8-2.3	diff-	nd	-	r	84	2
PR3Tg12D2	mec+ 3.4-7.9	diff-	nd	-	r	nd	nd
PR3Tg12D3	mec+ 3.4	diff-	diff-	-	r	nd	nd
Kd11BaD1	mec- 2	diff+	nd	-	r	89.1'	2
Kd11BaD3	mec- 1.3-3	nd	nd	nd	nd	nd	nd

cross-feeding or the selection of a non-hybrid variant.If the density was too low,cell-cell contact would be insufficient to allow for adequate membrane fusion and the production of hybrid cells.R5/3OA cells are capable of metabolic cooperation with other cells at a very low incidence.This could theorectically interfere with selection process by mutual rescue of cooperating PSA4 and R5/3OA cells.This should not,however have affected the isolation of hybrid lines as the selective medium was not introduced to the cells until 24 hours after the fusion treatment by which time the majority of heterokaryons should have formed and no evidence of survival of either PSA4 or R5/3OA cells was found when these were plated out in coculture in conditioned medium containing HAT and Ouabain.

A second obstacle to the isolation of viable hybrids from these two ec cell lines was their apparent sensitivity to the fusogen treatment which seemed to be causing the early death of putative heterokaryons either before mitosis could occur or immediately afterwards.Multinucleate cells were visible in culture dishes for the first three days post-fusion but after this time they disappeared.It has been demonstrated that the behaviour of heterokaryons can be markedly different from their resultant hybrid cells [1.6.] and the failure of these cells to make the transition from a binucleate cell to a hybrid one could be a result of any one of a number of factors.

It is proposed that a heterokaryon would be unable to produce a viable hybrid if; i) The heterokaryon was unable to survive,or unable to undergo mitosis in the culture conditions available, ii) The hybrid cell was more sensitive to the culture conditions than the heterokaryon and was therefore unable to support its growth after the mitosis event, iii) Both heterokaryon and hybrid cells had more stringent growth requirements than either of the two parent ec lines and therefore could not survive in the culture medium provided,iv) The two lines R5/3OA and PSA4 were incompatible in hybrid form,v) Either the fusogen treatment,trypsinisation or the combination of these caused the cells to be so traumatised as to become inviable.

However hybrid lines have been isolated from ec cells before either in combination with other ec cell lines [I.6.] or with other types of cells [1.6.].

Previously it has been found that cell lines selected for a cooperation deficient phenotype [1.7] have also tended to have a reduced developmental

capacity.This has led to speculation that the two properties are at least partially linked to each other.

The isolation in this study of cell variants with mec- diff+ and with mec+ diff- phenotypes greatly weakens this argument and suggests that the two processes are genetically independent of one another.

The preceding three chapters of this study have reported the isolation and characterisation of a series of hybrid and hybrid-derived variants whose properties are summarised in Table 6:1.

Results show that the fusion of a mec- diff- (cooperation deficient line which is defective in spontaneous *in vitro* differentiation and does not form differentiated tissues in tumours) ec cell line (R5/3OA) with a mec+ diff+ (capable of metabolic cooperation with a high probability and forms differentiated tissue both *in vitro* and *in vivo*) line (PSA4) yields hybrid lines (PR3,PR3/4) which are both pluripotent and homotypically mec+.

This experiment therefore shows that in these lines the mec+ and diff+ phenotypes are dominant (or at least semi-dominant as both cooperation and differentiation capacity appear to be slightly reduced in PR3).This lends support to the proposition that these changes are genetic in nature and that the lesions responsible for these two defects are both recessive and so in the hybrid form give the wild type phenotype.A genetic origin has previously been indicated for the mec- lesion by the complementation of two cooperation deficient cell lines to give a cooperation competent hybrid [1.7].However neither of these results exclude an epigenetic control of these properties,although gene expression is less likely to be affected in the fusion of two ec cells than for example,if a terminally differentiated cell was fused with a pluripotent line [1.6.].

Assuming dominance of the plus allele in the case of HPRT,mec and diff the deficient phenotype of any of the three (isolated from the hybrid line) would be a result of loss of the wild-type gene presumably by loss of the chromosome on which it is carried.

Both of the lines isolated in this study and identified as hybrids have subhexaploid karyotypes and two metacentric marker chromosomes,strongly suggesting that both lines originate from one or more fusion events involving a single R5/3OA cell and a single PSA4 cell.Further analysis of the trypsin banded

karyotypes of these lines and a comparison with the trypsin banded karyotypes of the parent lines should confirm this view.

The range of chromosome counts of the original hybrids PR3 and PR3/4 is noticibly larger than the range of either of the two parent lines.Tissue culture lines,particularly of tumour origin,tend to be aneuploid and their chromosome count tends to vary from cell to cell.However both of the parent lines show a clearly identifiable modal number of chromosomes with the deviation to either side dropping sharply.

The wider spread of chromosome counts of the lines PR3 and PR3/4 can be accounted for by two explanantions which are not mutually exclusive.These are chromosome loss,which is expected to be of the order of 15% [1.6] in a newly formed hybrid line and heterogeneity in that hybrid variants resulted from more than one fusion event.

Chromosome loss would theorectically lead to a skewed range of chromosome counts with the mode moved to the left.There is little evidence of skewing of either of the two original lines and although chromosome loss is evident from some variants (notably those isolated to be diff- and the clonal derivative Kdla.6) the chromosome content of PR3 does not appear to be particularly unstable even after it has been passaged *in vivo* in the form of the tumour derived line PR3Tg12TX.

Heterogeneity of the PR3 cell population seems likely both because cells of the fusogen treated monolayer were pooled with no attempt made to clone them before the isolation of variants and also because cell variants isolated subsequently from this cell population all have a narrower range of chromosome counts than the parent line.

However the number of fusion events is probably not very high as is indicated by the isolation of the diploid line PR5 which has a chromosome count profile almost identical to that of PSA4 and appears to be a Ouabain resistant derivative of this line rather than a hybrid.This line did not appear to be contaminated by a subpopulation of any other cells such as would be expected if small numbers of hybrid cells were present indicating that the incidence of viable fusion is quite low.

The accidental isolation of this diploid line has proved very useful as it has

been used as an internal control for the effects of PEG 1000 fusogen on the phenotype of the mec+ differentiating line PSA4.No major differences in this line as compared to PSA4,with the exception of ouabain resistance,were noted although the large amounts of haematopoietic tissue found in tumours of this line were slightly unusual indicating that the fusogen may have some effect on the differentiative capacity of the line.This effect was however minimal and productive bone marrow was also found in small quantities in tumours derived from the PSA4 line.

The isolation of variants from the hybrid line PR3 has indicated that all of the combinations of locus involving ouabain resistance,HPRT enzyme,cooperation and differentiation *in vitro* are segregating independently of each other.However,on the basis of these experiments,the *in vivo* differentiation deficient (dift-) phenotype cannot be separated from the absence of the HPRT enzyme (HPRT-).If this association were found to be real it would indicate that the X- chromosome has a locus (or loci) which is involved in the *in vivo* differentiation of mouse teratocarcinomas.At present this proposition is based only on the results of one cell variant,PR3Tg12, and therefore cannot be taken as proof of any kind of genetic association between the two phenotypes.

An alternative,although unlikely proposal,would be that the HPRT- phenotype is incompatable with *in vivo* differentiation.It is difficult to envisage how the two phenotypes could be physiologically connected and the study of other mutants possibly linked in a dift-/HPRT+ fashion and the isolation of crossover mutants should help clarify this situation.

The parent line of these two cell variants PC13 and its thioguanine derivatives PC13Tg8 and PC13Tg7 all have a dift+ phenotype although this is restricted when compared to the primary tumour and has tumours which are predominantly of ec cell type with a high incidence of nervous tissue.It is proposed therefore that a dift- lesion connected with the X- chromosome would be created by a mutation in the PR3Tg8 line from which R5/3 is derived (evidence that this is plausible comes from the finding that high passage cells of this line were found to have lost their developmental capacity).The finding that both the mec- line R5/3(from which R5/3OA is derived) and the mec+ revertant of R5/3 (H2T12) are both HPRT- and Dift- is consistent with this origin.

Finally the linkage could be entirely fortuitous and the further investigation of HPRT- variants of PR3 may reveal that this is the case.It would seem worthwhile however to investigate the tumourigenic potential of the other 17 thioguanine resistant variants (PR3Tg1-PR3Tg20) isolated from the hybrid line PR3 as a preliminary to further investigation of this property.

If the dift and HPRT loci were found to be linked this would have important implications for the use of HAT selective medium and drug resistant mutants in the investigation of differentiation.

If a locus involved with differentiation was found to be linked to the X-chromosome it could prove to be of significant value in elucidating the early mechanisms of cellular differentiation and X-inactivation,which in this respect at least would be necessarily connected to one another.X-inactivation in the early embryonic and extraembryonic tissues has already been shown [1.3.] to be different in nature to that found in tissues of the older embryo and of the adult mouse.

The isolation of cooperation deficient (mec-) hybrid lines from the thioguanine resistant (mec-,dift-) line PR3Tg12 is also reported in Chapter 4 and it has been found that those derivatives investigated (Kdla and Kdla.6) are all capable of *in vitro* differentiation as measured by the extent of endoderm differentiation and cavitation formation in suspension aggregates (embryoid bodies) and this is also true for the line KdllBa which gave ambiguous (mec+ and mec-) results in uridine transfer experiments.

However the extent of *in vitro* differentiation in all of the lines PR3Tg12,Kdla,Kdla.6 and Kdllba is reduced in comparison to the original hybrid PR3 which itself shows less differentiation in EBs than the diff+ line PSA4 from which it was derived.

This reduction in differentiation capacity may be related to the difv- phenotype in the case of PR3Tg12,kdla,kdla.6 and Kdllba or it may be entirely unrelated.The slight reduction of *in vitro* (and probably also *in vivo*) differentiation which was observed in the hybrid line PR3 may be due to the increased ploidy of this line or other trivial explanations connected with fusogen treatment or with the hybrid state.

Another explanantion could be that all or some of the loci responsible for the

diff+ phenotype are not completely dominant and so interact with the diff- genotype of R5/3OA to produce some restriction of differentiation potential.The diff+ phenotype is in any case likely to be the result of a complex series of loci and probably also epigenetic factors.

McCue [108] has shown that the phenotypes of a series of hybrid lines created by fusing two independent mutant differentiation defective cell lines which failed to differentiate in reponse to RA(retinoic acid) or HMBA (hexmethylene bisacetamide) suggest that the mutations involved are both complementary and recessive.This finding suggests that more than one loci is involved in the control of differentiation.A further class of hybrids [101] derived from diff- mutants were found to be capable of differentiation *in vitro*(dif b+) but not *in vivo*(dift-) providing further support for the distinction between *in vitro* and *in vivo* forms of differentiation.

As with previous cooperation deficient lines which have been called Mec- ,these hybrid variants (and the parental line R5/3OA) are all capable of low probability interaction with other cells of the same line (homotypic cooperation).

Therefore although these results seem to exclude the R5/3OA cooperation deficient lesion from a role in reduced developmental capacity they do not exclude the possibility that a completely cooperation deficient line may be unable to differentiate and one proposed role for these hybrid cooperation deficient lines is to use them as a basis for the isolation of completely defective cooperation mutants using further kiss of death selection and with the aid of mutagenesis.

Neither do these results formally exclude involvement of the mec- lesion from a role in *in vivo* differentiation in that none of the mec- lines isolated in this study are able to differentiate *in vivo*.The isolation of a mec- mutant which can differentiate *in vivo* would substantially strengthen the argument that cooperation and differentiation are independent of one another.

It has also been shown in this study that it is possible to select for hybrid cell variants with reduced *in vitro* differentiative capacities on the basis of the morphology of their colonies when plated onto gelatinised tissue culture dishes in STO feeder conditioned medium.Both of the lines tested (PR3Tg12D1 and PR3Tg12D3) which were isolated in this way are unable to differentiate either *in*

vivo or *in vitro* lesion.This phenotype would be expected if the parent (PR3Tg12) lines inability to differentiate were due to the loss of a gene (or genes) by the mechanism of chromosome loss.

No tumours have been obtained from the line PR3D1,which is in any case capable of substantial *in vitro* differentiation,but it would be interesting to discover whether or not this line (derived from a dift+ parent) is also able to differentiate in mouse tumours.

With the exception of the line PR3Tg12D1.1 which is mec-and diff-,all of the lines selected for reduced differentiation capacity behave as the lines from which they were isolated in that they are mec+ in the case of lines selected from PR3Tg12,and mec- in the case of lines selected from KdIIB1.This further supports the postulate of independent segregation for the two properties.The isolation of a mec- and diff- line (PR3Tg12D1) means that it may be possible to identify the chromosomes involved in these lesions by identifying the chromosomes present and absent in each of the segregant lines using trypsin banded karyotypes.

An analysis of the trypsin banding of the chromosomes of all of these lines should allow the identification of the chromosomes whose absence is responsible for the mec- lesion,and the lesions responsible for defects in both *in vivo* and *in vitro* differention.Considerable chromosome loss was observed in the selection of the lines isolated for deficient *in vitro* differentiation indicating that several genes (chromosomes) are involved,or that several copies of these chromosomes are present in the original hybrids.This contrasts with the relatively low level of loss observed in the karyotypes of hybrid variants selected for reduced cooperation.

CHAPTER 7

ISOLATION AND CHARACTERISATION OF A CELL LINE DERIVED FROM THE INNER CELL MASS OF A MOD1^{NULL} MOUSE BLASTOCYST

This Chapter deals with the development of a method for the isolation of cell lines from mouse blastocyst embryos,and with the characterisation of one such cell line,Mod1.The mice used as the source of the blastocysts were of C57Black background and contained a mutation which makes them deficient in the production of L-Malic enzyme [personal comm. ProfessorGardner].This line is known as C57/Bl Mod1^{null} (also abbreviated to Mod1^{null}).The mice were obtained from Professor R.L.Gardner in Oxford.

Some cell lines isolated from mouse ICM cells have previously been shown to have an ec cell phenotype [1.5.3] and therefore the ec cell line was used during the characterisation of Mod1 as a control for this cell type.PYS,a cell line derived from a parietal yolk sac carcinoma,has a parietal endoderm (PE) phenotype and so was used in conjunction with the ec cell control (PSA4) during characterisation of Mod1,because of the morphological resemblance to PE which "on-feeder" monolayer cultures of Mod1 cells display.

7.1. Isolation of Cell Lines From Mouse Blastocysts

Several attempts were made to isolate cell lines from mouse blastocysts derived from both Mod1^{null} and from Tcd^{sey} mice, using published methods [37,99 and 17].These methods are described as #1 and #2 in Table 7:1 and are described in detail in Chapter 1 [1.5.3].None of these experiments yielded any permanent cell lines,although in most cases outgrowths of cells were observed initially growing around the original mass of embryo cells.These outgrowths however only survived the first few days in culture and then appeared to die out.

The method described in Table 7:1 as #1 required immunosurgery of the blastocyst embryo to remove the outer layer of trophectoderm which surrounds the inner cell mass cells.Therefore sera from various sources were tested for their ability to lyse mouse red blood cells,and a source of anti- mouse antibody and a source of complement were found. The results of these

Table 7:1

A SUMMARY OF THE METHODS AND MODIFICATIONS EMPLOYED IN THE ISOLATION OF CELL LINES FROM MOUSE BLASTOCYST EMBRYOS

Summarises the original methods and the modifications to those methods neccessary in the course of isolating cell lines from C57Bl.Mod1^{null} mouse blastocyst embryos.

Method	Source	Treatment prior to plating out	Plating out method and medium used	References	Results, Remarks and comments
1	Mod1(null) early blastocyst embryos (10 in all).	i)overnight incubation of embryos to give a fully expanded blastocyst. ii)pronase removal of the Zona Pellucida(ZP) iii) Immunosurgery	Plated out individually into 35mm cloning wells containing STO feeder layer.Medium supplemented with PSA4 conditioned medium concentrate.	Martin,G.R. PNAS(usa) 78 (1982),7634-7638.	Outgrowths observed but all died out before sufficient cells for a frozen stock could be recovered.
2	Early blastocyst emoryos.	Overnight incubation and removal of ZP as in 1(i) and 1(ii).	Plated directly into 35mm cloning wells with STO feeder layers. 20% FCS and B-Mercaptoethanol supplemented medium. Pick off at egg cylinder stage and transfer to fresh wells.	Evans,M.J. & Kauffman,M.H. Nature 292 (1981),154-156.	Outgrowths formed but no permanant lines were obtained.Cells died at the transfer from the egg cylinder stage.
3	Early blastocyst embryos.	Overnight incubation. Removal of ZP. Immunosurgery.	Plated into 35mm ("Optikon") cloning wells with STO feeder layer. 20% FCS and B-Mercaptoethanol in medium.Medium replaced only 50% at a time.	Methods 1 and 2	Outgrowths but no cell lines.
4	Early blastocyst embryos.	a) No Treatment b) Overnight incubation removal of ZP and immunosurgery.	Plated into 35mm ("Optikon") cloning wells with STO feeder layer. 20% FCS and B-Mercaptoethanol in medium. Very small volume of medium used (10ul) and replaced half at a time.	Methods 1,2 and 3.(see also Axelrod Dev.Biol.101 1984,225-228)	4a) Isolated the lines Mod1.2, Mod1.3,Mod1.4,Mod1.5,Mod1.7 and Mod1.8. 4b) Isolated the line Mod1

experiments are summarised in Table 7:3 and show that the IgG anti-mouse antibody (Serum 1 in Table 7:2b),raised in rabbit injected with mouse cells by Dr W.McBride,was effective against mouse red blood cells at a dilution of at least 1/16.

Similarly both the complement present in human blood (SB) and the complement found in Guinea pig serum obtained from SAPU (Scottish Antibody production Unit) were effective in lysing those cells with which the antibody had reacted.Since the supply of GPS was more readily obtainable,this was used in all further experiments.As the results in Table 7:2 show,there was a slight reaction of the GPS with mouse blood cells at normal concentration which probably indicates a low titre natural antibody,and so GPS was used at a concentration of at least 1/8 when it was shown to still be fully effective.Serum obtained from my own blood (JS) was found to contain a high titre anti-mouse antibody (detectable at a 1/64 dilution,[Table 7:2a]) and therefore could not be used as a source of complement in these experiments.

Once a suitable antibody and source of complement had been found,the system was tested on mouse blastocyst embryos,using indirect immunofluorescence to detect the presence of the antibody on the outer surface of the embryo.As the results shown in Table 7:4 indicate the anti-mouse cell antibody appears to attach to the outer layer of cells of the mouse blastocyst embryo and does not appear to penetrate into the inner cells.These observations together with light microscope observations that both a reduction in size and collapse of the blastocoel occurs when the blastocysts were treated with the antibody and lysed with complement,were taken to indicate that the immunosurgery was effective and that the inner cell mass was being dissected cleanly out of the blastocyst.

Cell lines were obtained using various modifications of published methods [Table 7:2] which were designed to maximise the growth of inner cell mass cells.This was done both by using culture medium supplemented with medium extract taken from medium conditioned by feeder dependent and pluripotent ec cells (PSA4 were used in these experiments) [99] and by enriching the culture medium using B-Mercaptoethanol(10^{-4}M) and 20% foetal calf serum in place of the usual 10% FCS [17,and see also enriched medium section in 3.1.6].The use of such media allows the survival of the inner cell mass cells in the absence of the trophectoderm and supports the proliferation of cells in "outgrowths" which

Table 7:2

AGGLUTINATION AND LYSIS TESTS TO ESTABLISH THE EFFECTIVENESS OF ANTIBODIES AND COMPLEMENT SOURCES USED IN IMMUNOSURGERY TECHNIQUES

a) Result of incubating mouse red blood cells at 37°C with serum derived from one of two rabbits to investigate their natural anti-mouse antibody levels and with human serum (JS) containing complement which was also found to have a high titre mouse antibody and so was useless as a source of complement in combination with an antimouse serum

b) Result of incubating mouse red blood cells at 37°C with:

1 Serum serially diluted in PBS
2 Complement derived from Guinea pig serum (GPS) serially diluted plus 0.1ml of serum 1
3 Human complement (SB) serially diluted plus 0.1ml of serum 1
4 GPS serially diluted with no added serum 1

Each well was made up to 0.3ml with PBS (saline solution) and then incubated with 0.1ml of mouse rbc concentrate.Three "saline only" control wells were also set up and showed no reaction after this incubation.Wells were scored for a reaction with blood cells as follows:

Agglutination - A
Lysis - L
No reaction - N
Both A and L - A+L

a)

Sample	Dilution at which antibody is effective	Dilution at which complement is effective	Remarks
Rabbit 1	1/32	-	Moderate level of natural anti-mouse. Probably IgM as it gives poor Agglutination, Good lysis.
Rabbit 2	1/16	-	low level natural anti-mouse. Probably IgM.
Complement (Human - JS)	1/64	1/8-1/10	High titre anti-mouse antibody. Renders complement usless in combination with anti-mouse antibody for immunsurgery as complement is only functional at dilutions of 1/8-1/10.
Saline (PBS)	-	-	Serves as a control. No agglutination or lysis observed. The saline used is therefore isotonic with the mouse red blood cells.

b)

Experiment	Well Dilution N	N/2	N/4	N/8	N/16	N/32	N/64
1 serum 1	A+L	A+L	A	A	N	N	-
2 GPS	L	L	L+A	A+L	A+L	A+L	A+L
3 SB	L	L	L+A	A	A	A	A
4 GPS-no ab		N	N	N	N	N	N

notes:

i) ab = antibody (serum 1)

ii) Serum 1 (derived from rabbit) shows agglutination up to a dilution of N/16 (and so was used at this dilution in immunosurgery experiments.

iii) Active complement was found in GPS and causes lysis in combination with serum 1. At undiluted levels (experiment 4) lysis occurs indicating the presence of low level natural anti-mouse antibody. This complement source was therefore used at a dilution of 1/4.

Table 7:3

USE OF INDIRECT IMMUNOFLUORESCENCE TO DETERMINE THE SPECIFICITY OF AN ANTI-MOUSE CELL ANTIBODY AND THE EFFECTIVITY OF COMPLEMENT

The Zona Pellucida of a series of ex panded mouse blastocysts was removed and the resulting embryo incubated in four different combinations of antimouse serum,complement and FITC linked anti-rabbit sera.

Results show that the incubation of an embryo with either the anti-mouse sera or the FITC anti-rabbit sera alone did not yield any fluorescence on the embryos.Incubation of the embryo with both antibodies together gave fluorescent embryos indicating that the mouse antibody was attached to the outer layer of the embryo and that the fluorescent antibody was attached to this antibody.If embryos treated with both antibodies were than washed thoroughly and incubated in serum containing complement the embryos lost both their fluorescence and their characteristic shape.This last result indicates that the immunosurgery technique was effective both in removing the outer TE cells and in protecting the inner ICM cells from antibody attack.

FITC - Fluoro-Isothiocyanate

TE - Trophectoderm

ICM - Inner Cell Mass

Experiment	Result
Embryo + FITC -anti-rabbit	No Fluorescence on embryo
Embryo + anti -mouse serum	No fluorescence on embryo
Embryo + anti -mouse serum + FITC-anti-Rabbit	Outer cells of the embryo (TE) show green fluorescence. no visible fluorescence of the inner cell mass.
Embryo + anti -mouse serum + FITC-anti-rabbit. washed thoroughly and lysed with complement.	No fluorescence of the mass of remaining cells (ICM). Fluorescent debris.

Table 7:4

SUMMARY OF CELL LINES ISOLATED FROM MOUSE BLASTOCYST EMBRYOS AND FROM BLASTOCYST DERIVED CELL LINES

Lists the cell lines isolated using methods outlined in Table 7:1 of this chapter and the derivatives of one of these lines (Mod1).

Cell Line	How Isolated	Derivation
Mod1	Method 4a (Table 6:1)	Single expanded blastocyst from a C57Bl.Mod1(null) mating.
Mod1.2	Method 4b (Table 6:1)	Individual expanded blastocyst from a C57Bl.Mod1(null) mating.
Mod1.2	"	"
Mod1.3	"	"
Mod1.4	"	"
Mod1.5	"	"
Mod1.7	"	"
Mod1.8	"	"
Mod1a	Cloning of Mod1.	Mod1
Mod1b	"	"
Mod1c	"	"
Mod1d	"	"
Mod1e	"	"
Mod1f	"	"
Mol.01	Culture of Mod1 cells	Mod1

form around the mass of ectoderm cells.

However it was found that the survival of cells derived from the inner cell mass was dependent,not on the presence or absence of the trophectoderm or of conditioned media but on the quantitity of medium used to plate out the embryos onto their feeder layers.

It was found that if 10µl or less of 20% FCS enriched medium was used then cell lines could be isolated from embryo outgrowths either with or without the presence of the trophectoderm.This finding has also recently been reported by H.Axelrod (6,5),who selected pluripotent (EK) lines from mouse embryos using this method.

Thus the cell line Modl was isolated from an outgrowth of a single ICM derived from a C57BL Mod^{null} embryo,cultured on a STO feeder layer in low volumes of medium.The cell lines Mod1.2,Mod1.3,Mod1.4,Mod1.5,Mod1.7,and Mod1.8,were all isolated from whole embryos from the same source and cultured as for Modl [Table 7:4].

Where applicable,that is when the immunosurgery technique was used,it was found that the use of pronase (0.05%) [117,see methods section 2.6.4 for protocol] was far more effective and reliable a method of removing the zona pellucida (ZP) of the embryo than the use of Acid salt solutions such as "Acid Tyrodes" solution [146].This latter was found to be inconsistent and frequently failed to remove the ZP even after 15-30 minutes incubation,although it did alter the morphology of the blastomeres,which became more rounded and separate from one another,as seen by the light microscope.Embryos not treated with immunosurgery were plated directly into the cloning wells because it was shown [Figure 7:1] that they were capable of hatching from the ZP without pronase digestion.

It was found that while outgrowth cells were still at low density that they stood a better chance of reaching confluence if only half of the medium in which they were growing was replaced at any one time,and this system was followed for growing up to the initial frozen stock of all of the lines isolated sucessfully from mouse blastocyst embryos.

Figure 7:1 shows mouse blastocyst embryos after 2 days [7:1a] and 3 days [7:1b] in culture on STO fibroblast feeder layers plated into 35mm cloning wells.

Figure 7:1

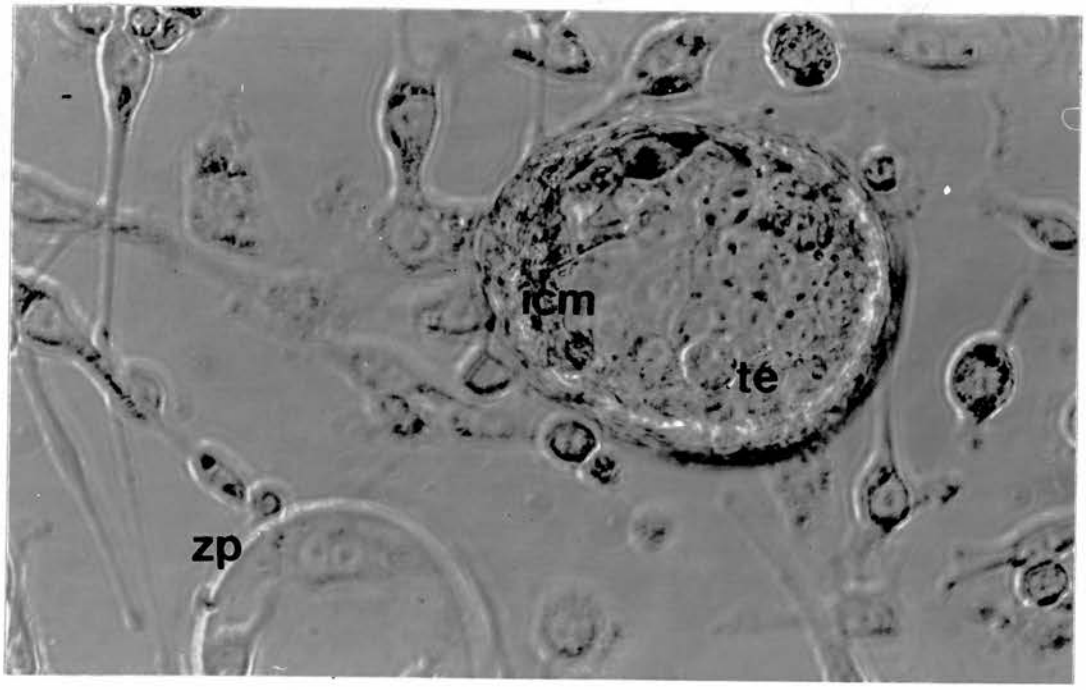
MOUSE BLASTOCYST EMBRYOS GROWING IN CULTURE IN STO FEEDER CELLS

Phase contrast microscopy of mouse blastocysts in culture **a)** 2 days and **b)** 3 days after they were flushed out of the uterus.(Magnification = 1600)

The blastocyst illustrated in **b)** has hatched out of its Zona Pellucida (zp) which can be seen at the bottom of the picture.

f - fibroblast feeder cell (STO)
zp - Zona Pellucida
icm - Inner Cell Mass
te - Trophectoderm


f
icm


icm
te
zp

The blastocyst shown in 7:1a still has its ZP while that in 7:1b has "hatched out" of its ZP which can been seen to the lower right hand corner of the picture.

7.2. Growth Requirements and Morphological Features of the Cell Line Modl

7.2.1. Growth Requirements

Modl cells [Figure 7:2] were found to grow only in medium containing at least 20% FCS and supplemented with 10^{-4}M B-mercaptoethanol.They are feeder dependent and were therefore routinely grown in small tissue culture bottles which had been pre- seeded with I0 ^{6} mitomycin treated STO cells [2.2.4].Recently it has been found possible to grow Modl cells in the absence of STO feeder cells,in medium pre-incubated with BRL cells [7.6.3] and supplemented with B- Mercaptoethanol (10^{-4}M) and 20% FCS.EC cells grown under these conditions show an absolute requirement for B- Mercaptoethanol [A.G.Smith per.comm.] but the role of the feeder layer has been replaced and the cells can be seeded onto tissue culture dishes pre- treated with 0.1% gelatin [2.2.4] without the need for feeder cells [Figure 7:2].In addition to the culture conditions described above it is also possible to seed out Modl cells at high density in the absence of both BRL conditioned medium or feeder cells and obtain cell growth,however this causes various morphological changes and is described in a later part of this chapter [7.6.1].

Modl cells can be subcultured in the way routinely employed for ec cells and described in the Methods [2.2.3] using TVP which contains trypsin.However because of the large quantities of extracellular matrix which are secreted by Modl cells,this method was not very satisfactory as it tended to give messy cultures containing lumps of partially digested matrix.Therefore Modl cells are now routinely sub-cultured using EDTA only which is sufficient to remove most cells from their growing surface [2.2.3].

When grown in medium supplemented with 20% FCS and 10^{-4} B-mercaptoethanol on an STO feeder layer Modl cultures were subcultured approximately once every 3-4 days (split ratio of 1/10) and required a medium change about once a day.

Figure 7:2

MORPHOLOGY OF MOD1 CELLS IN CULTURE WHEN VIEWED BY PHASE CONTRAST MICROSCOPY

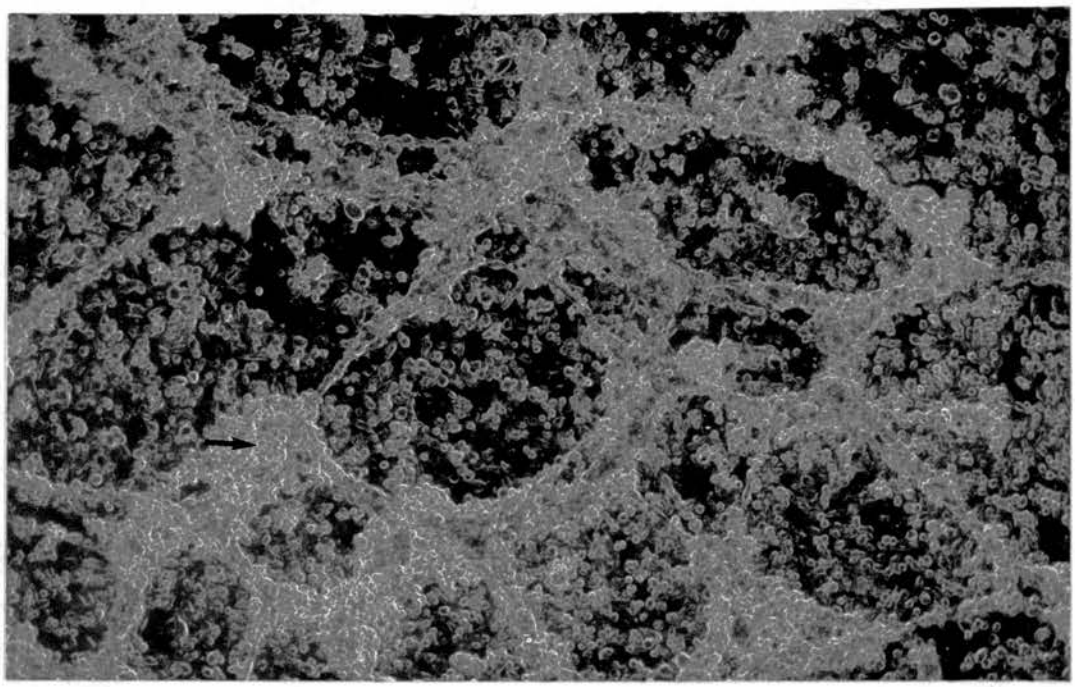
Shows the morphology of a Mod1 monolayer culture growing on an STO feeder layer as viewed by phase contrast microscopy.

a) Low power (128) view showing the characteristic formation of extracellular matrix secreted by the cells and their typical rounded appearance.Clumps of cells attached to matrix (indicated by an arrow) are connected by strands of matrix and cells.Other cells,apparently not attached to matrix (small arrow) grow in the spaces.

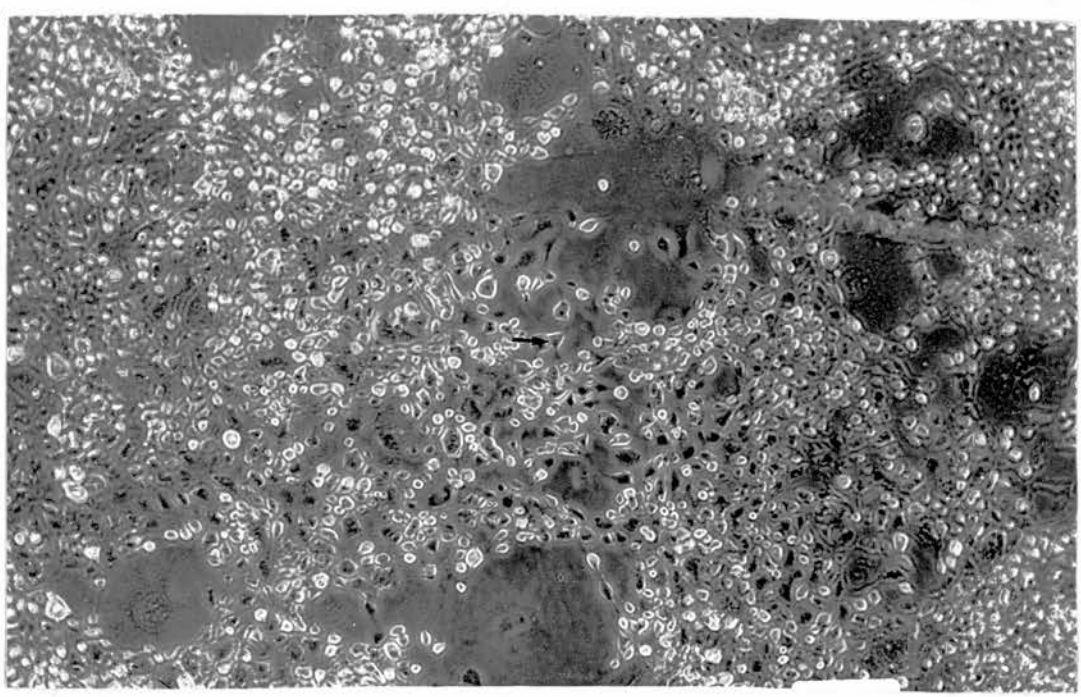
b) Illustrates the same Mod1 monolayer culture as in **a)** but at a higher magnification (320) showing the characteristic rounded formation of the majority of the cells and the "triangular" shape of those cells properly attached to the dish (arrow).

c) High power magnification (1024) of a low density culture of Mod1 cells growing on an STO feeder layer illustrating the variety of morphological types present under such conditions.

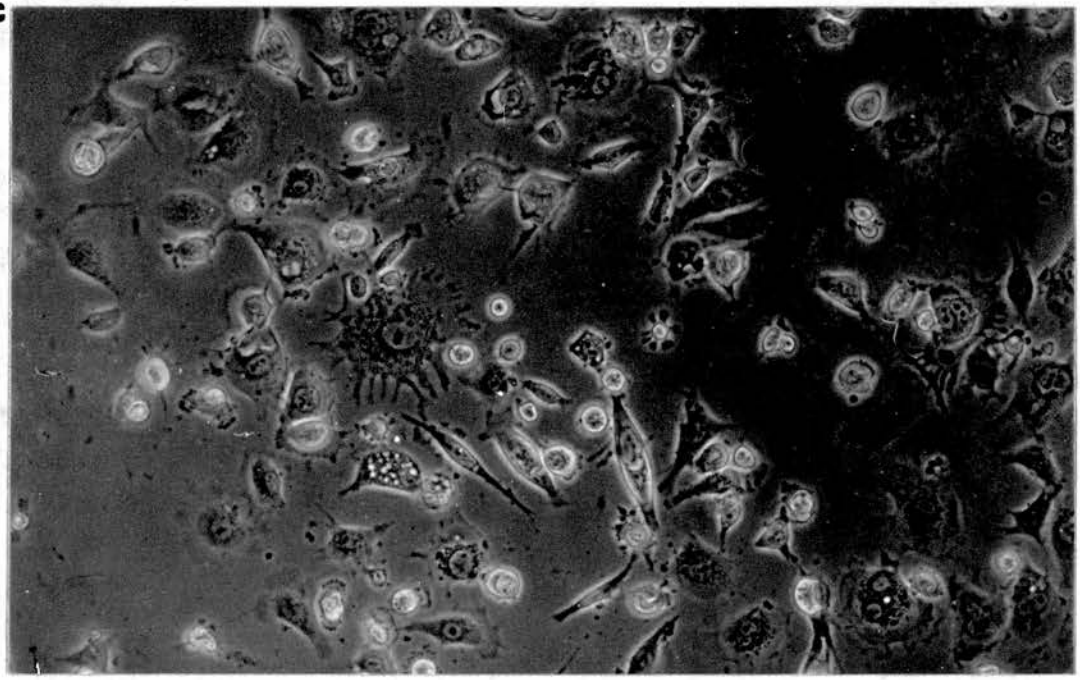
a



b



C



7.2.2. Cell Morphology - Phase Contrast Microscope

The morphological characteristics of the Modl cell line are summarised in Table 7:5.The cells are mostly rounded up in normal culture and smaller than would be expected of ec cells.They produce large quantities of an extracellular matrix on which the cells grow and which appears to physically prevent Mod1 cells from becoming confluent by becoming detached together with the majority of cells after reaching a certain density.

When plated out at low density the cells seemed to spread out more on the culture surface,form more cell-cell contacts and are less rounded,however when their numbers increased this situation is overcome and as their numbers grow and more matrix is produced they revert to the high density morphology described above.Modl monolayer cultures have also the tendency to produce rounded vacuolated structures which detach from the matrix and remain suspended in the medium.These resemble the characteristic vesicles produced by endoderm forming cells.

Figure 7:2 illustrates the characteristic growth of Mod1 cells in "on feeder" culture.Cells appear to grow along the matrix which they produce giving the cultures a characteristic "swirled growth pattern" in monolayer culture when viewed by low power phase contrast microscopy [Figure 7:2a].The vesicles which these cells also produce,and their rounded growth,give these cultures a "messy" appearance as though cell death were occuring although there is no evidence to suggest that this might be the case.Figure 7:2aii illustrates that these cells have a regular triangular shape and size similar in nature to that of cells of PYS cultures.Monolayer cultures of PYS cells however do not have the vesicles or matrix formation of Mod1 and do not require STO feeders for their low density growth.

7.2.3. Isolation of Mod1.01 Cells

A cell line (Mod1.01) has been isolated from cultures of Mod1 cells growing on an STO feeder layer.The majority of cells (including most STO cells) were removed when the extracellular matix reached "confluence" and detached from the culture bottle.The remaining cells were refed with enriched medium [2.3.4] and cultured for several weeks.

Table 7:5

A SUMMARY OF THE GROWTH REQUIREMENTS AND MORPHOLOGICAL FEATURES OF THE CELL LINE MOD1 IN COMPARISON TO THE EC LINE PSA4 AND THE PARIETAL YOLK SAC CELL LINE PYS

Illustrates the main morphological fetures of the Mod1 cell Line and indictes the equivalent features of the ec line and the endoderm cell line.Morphologically Mod1 cells have more in common with those of the endoderm line PYS and very little in common with the ec line PSA4.

Cell Line	Culture Requirements	Feeder Dependence	Growth in Conditioned medium in the absence of STO feeder cells	Cell type and morphology	Off feeder culture (differentiation)	Synthesis of extracellular matrix	E/M morphology
Mod1	20% FCS in CM B-Mercaptoethanol	Y	will not grow in STO conditioned medium grows in 75% brl medium in the presence of 20% FCS without a change in morphology	small rounded cells not in contact with each other, when in the absence of brl medium or STO feeders cells become more epithelial spread out and form contacts. Characteristic 'endoderm vesicles' form 1-2 days after plating onto feeder layers.	when grown in EC20 cells become very closely packed and spread out. Multinucleate cells are common. Reduction in the production of matrix.	Extracellular matrix secreted in large quantities. Trypsin labile.	majority of cells resemble PE or PrE. RER and secreting vesicles sparse microvillus distribution.
PSA4	10% NCS in CM	Y	will grow in both STO and 75% brl conditioned medium without differentiation	Epithelial ec cells	Differentiates to a large number of cell types when taken off STO feeder cells and grown in EC10 medium	No extracellular matrix synthesis observed	large nuclei. little cytoplasm with few organelles prominent nucleoli
PYS	10% NCS in CM	N	N/A	Regular, triangular shaped cells	N/A: i.e. morphology of cells is not affected by the presence or absence of STO cells	matrix does not appear to be secreted in monolayer culture	Resembles PE cells RER sparse microvilli

They were then subcultured,plated onto gelatin and a permanent stock frozen down.The resultant line Mod1.01 appears to have similar morphology to Mod1 [Figure 7:2b] but to be capable of growth in the absence of STO feeder cells.

7.2.4. Cloning of Modl

Because of the difficulties of culturing these cells at low densities,attempts to clone the Modl line met with little success for a long time,therefore all the characterisation was done on the parent (uncloned) line.Now however using the technique of culturing in very small volumes of medium,which was originally developed for isolating the parent line from blastocyst embryos,clones of Modl have been obtained and frozen down as permanent stock (Mod1a,Mod1b,Mod1c,Mod1d,Modle,Mod1f).Although these lines have not as yet been characterised in any great detail their morphological features appear similar to those of the parent line when plated on STO feeder cells.

7.2.5. Electron Microscopy of Modl and PYS cells and Comparison of Their Morphology

E/M of PYS cells has shown that these cells have a predominantly Parietal Endoderm (PE) morphology with dilated RER profiles,few surface microvilli,elongated mitochondria and basement membrane secretion [Figure 7:3).

The ultrastructural picture presented by Mod1 cells is however more ambiguous,although the cell morphologies appear to be predominantly of PE or PrE origin.The same features of dilated RER profiles,and golgi apparatus are present and active secretion is apparent.Most cells have elongated mitochondria and few surface microvilli and basement membrane secretion is evident between many of the cells.Other features which are more reminiscent of early VE include cells with numerous microvilli,electron lucent vacuoles and junctional complexes although the thin RER profiles and the apical orientation of elv and mv,characteristic of VE,were not observed.The absence of apical orientation may be due to the non-ordered state of the monolayer culture but is probably also indicates that fully developed VE cells do not form a substantial part of the Mod1 makeup.

Figure 7:3

ELECTRON MICROSCOPY OF MONOLAYER CULTURE OF PYS AND MOD1 CELLS

Illustrates the ultrastructure of Mod1 and PYS cultures (grown in monolayer culture) when viewed by electron microscopy.

A1: low power view (magnification 3000) of Mod1 cells illustrating the variety of sizes found.The smaller cells are dominated by the nucleus, while in the larger cells the nucleus is situated accentrically.

2: illustration (magnificaion 6000) of the indented nucleus of a larger cell from a Mod1 cell population.Numerous mitochondria are evident in the cytoplasm.

3: Higher power magnification (9000) of a Mod1 cell with an indented nucleus.

4:Illustration of idstended rough endoplasmic reticulum (RER) in the cytoplasm of a Mod1 cell.Many Mod1 cells had large areas of their cytoplasm occupied with such distended RER indicating that secretion was taking place. (Magnification = 1500)

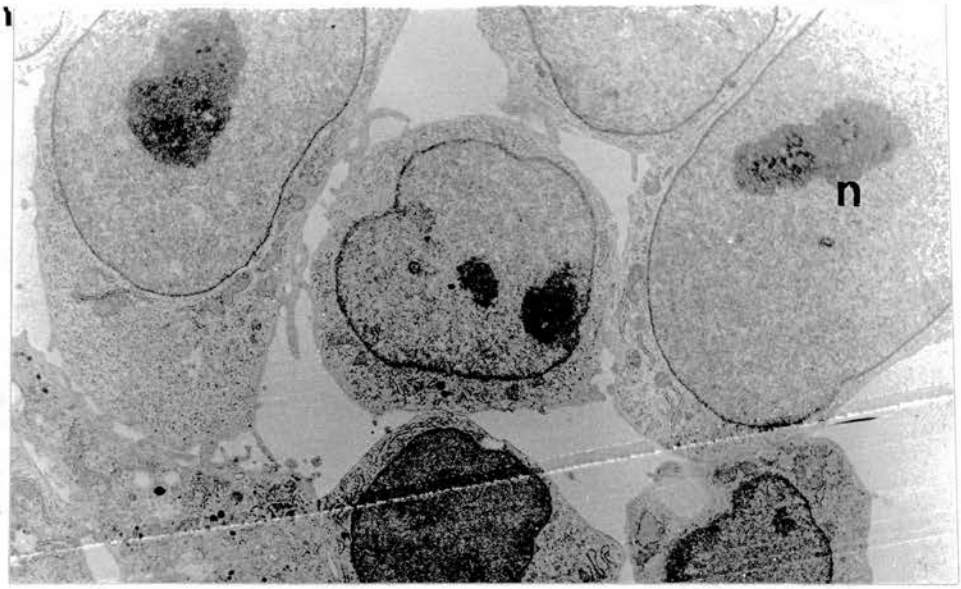
5: Intracellular space between two Mod1 cells.Arrow indicates matrix secretion (magnification 1500).

6: Illustration of Golgi body apparatus (G) in the cytoplasm of a Mod1 cell.(M) indicates a mitochondrion (magnification = 30000).

B1: Low power (magnification = 3000) illustration of PYS cells showing that the nucleus pedominates with the nucleus placed accentrically.

2: Illustration of distended rough endoplasmic reticulum (RER) which_is present in large areas of the cytoplasm of the majority of PYS cells examined (magnification = 22500).

A1



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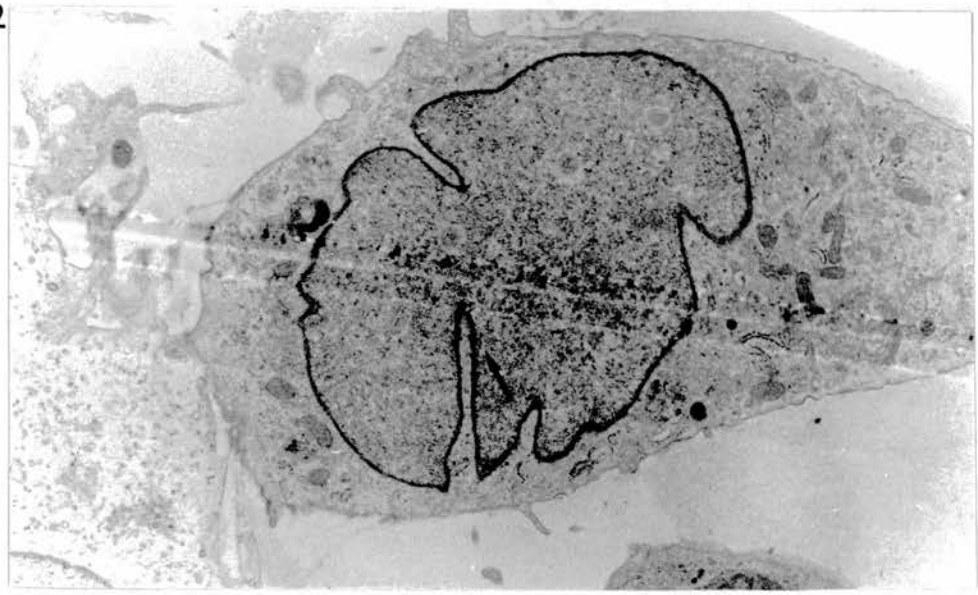

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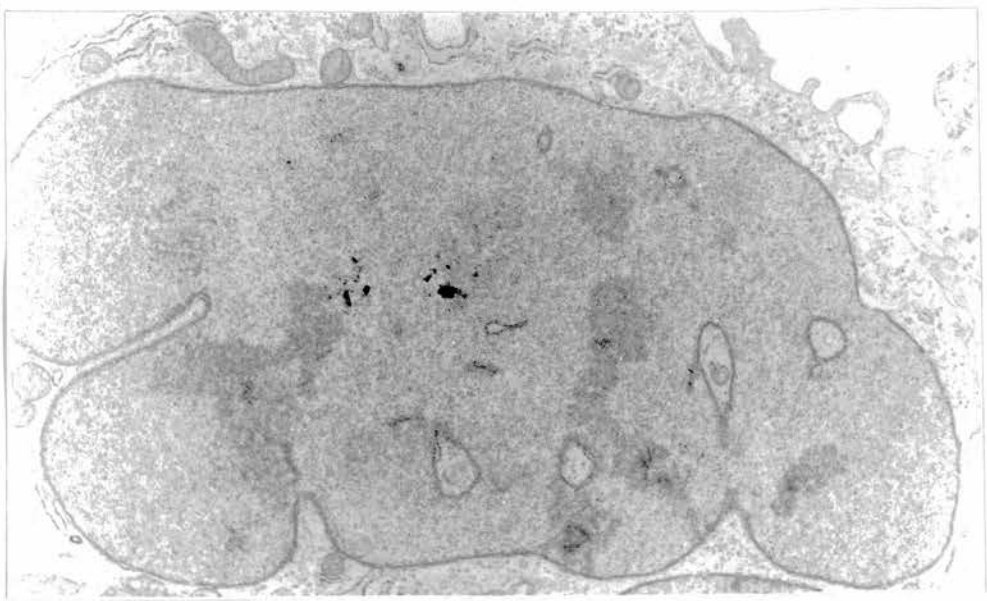

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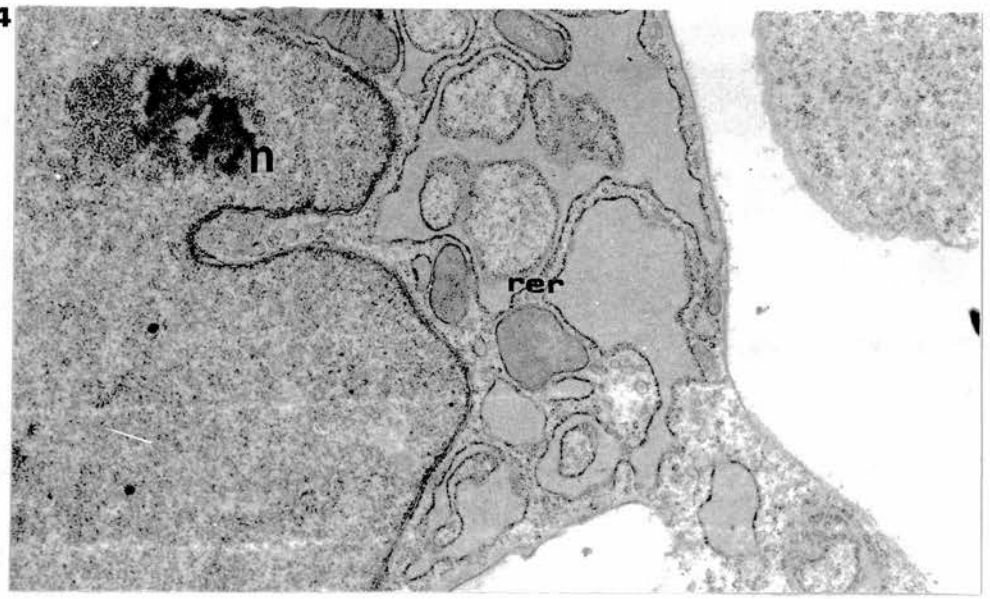

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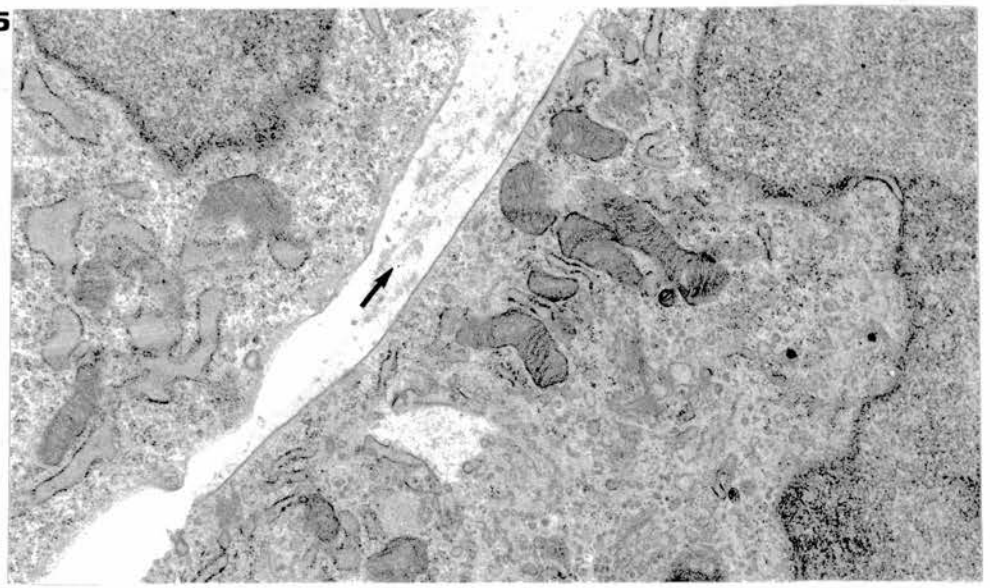

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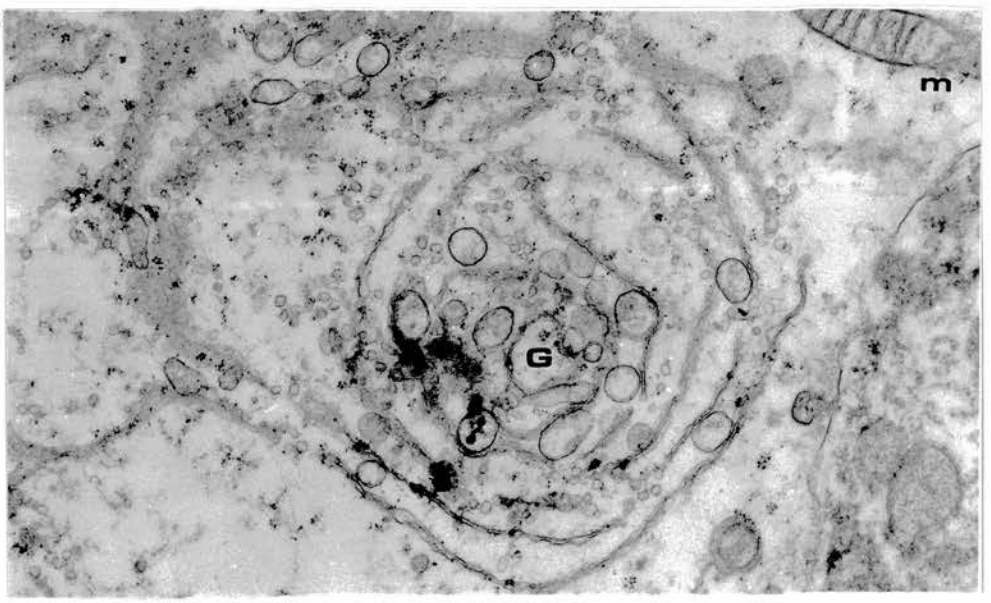
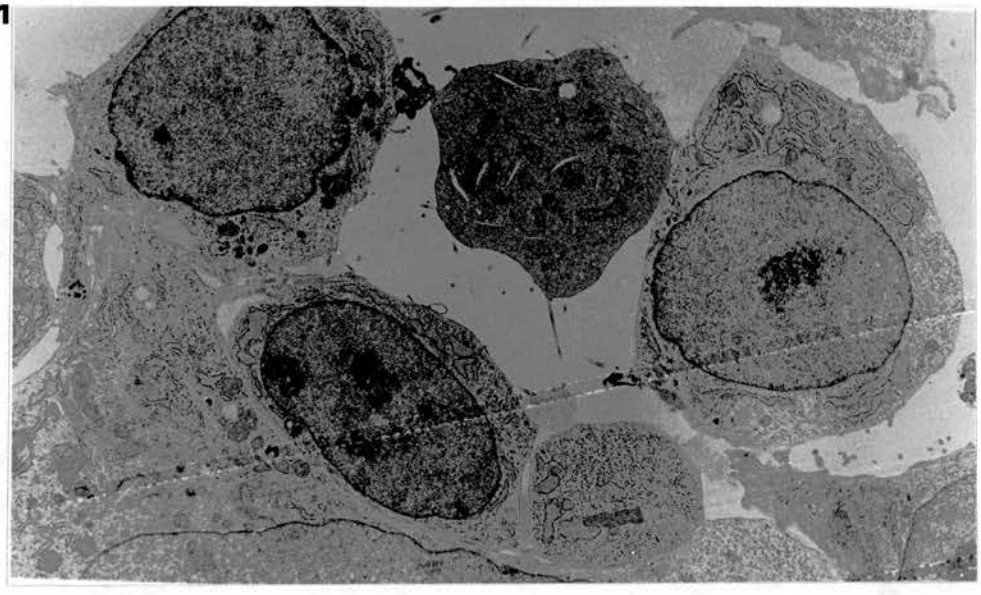


B1



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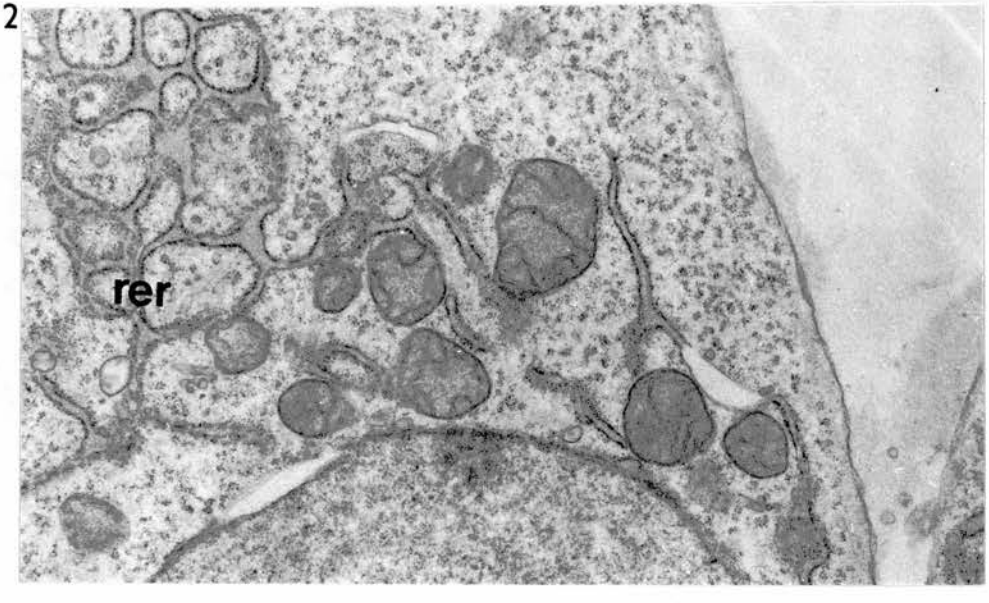


7.3. Karyotype of Modl

7.3.1. Chromosome Counts

Chromosome counts,shown in Figure 7:4,of metaphase spread preparations of Modl cells gave a modal count of 41.The majority (21/26) of counts fall into a category around this mode and the range within these 21 counts is very small.It therefore seems reasonable to conclude that the main karyotype of Mod1 is close to the normal mouse diploid karyotype.A small subset of tetraploid cells are also present which could be due either to the presence of mulitinucleate cells,metaphase block caused by the colchicine or to a small subset of cells within this non-cloned line which either have a genuinely different karyotype or have become polyploid by their inability to divide further as in the case of TE giant cells. The 41 chromosomes of the Mod1 cell line are all apparently normal,acrocentric mouse chromosomes [Figure 7:5].

7.4. Enzymatic and Biochemical Studies on Modl

This section deals with the various enzymatic and biochemical assays which were carried out on Modl cells,using PYS (a Parietal Yolk Sac derived cell line) and PSA4 (a pluripotential ec cell line) for comparison.The assays being designed to discover whether the properties of the Modl cell line resemble either the ec cell line or the parietal endoderm line.

7.4.1. Plasminogen Activator

The presence of plasminogen activator was tested for in two ways,the results of both methods being shown in Table 7:6a and 7:6b.The two assays fibrin gel overlay and fibrin gel are both described in the methods section of this thesis [2.7.2].Both methods exploit the role of fibrin in the blood clotting cascade reaction in which plasminogen has an enzymatic action.

The results of the fibrin gel assay [7:6a] demonstrate that Modl cells produce large amounts of plasminogen activator,this is compared with PSA4 cells which produce no PA and PYS cells which produce the enzyme but in lower quantities than those produced by the Modl cells.

Figure 7:4

ILLUSTRATION OF THE KARYOTYPE OF THE BLASTOCYST DERIVED LINE MOD1

Illustrates the apparently normal diploid karyotype (40 chromosomes) of the cells of Mod1.All appear to be accrocentric and there are no obvious differences from the normal mouse karyotype (see figure 1:2).Preparation was leishman's stained,Magnification = 5000).

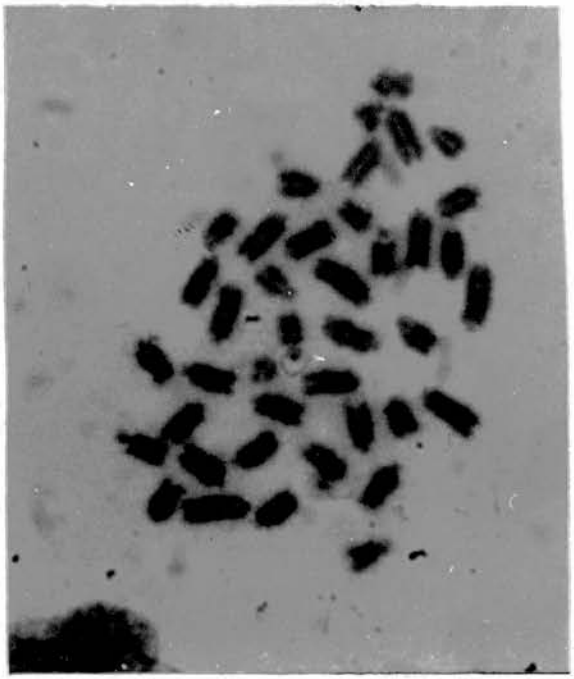


Figure 7:5

CHROMOSOME COUNTS OF CHROMOSOMES IN LEISHMAN'S STAINED PREPARATIONS OF METAPHASE SPREADS OF THE LINE MOD1

Illustrates that the modal chromosome count of cells from Mod1 is 41,and that the range is quite narrow (with the exception of a minority of tetraploid counts).

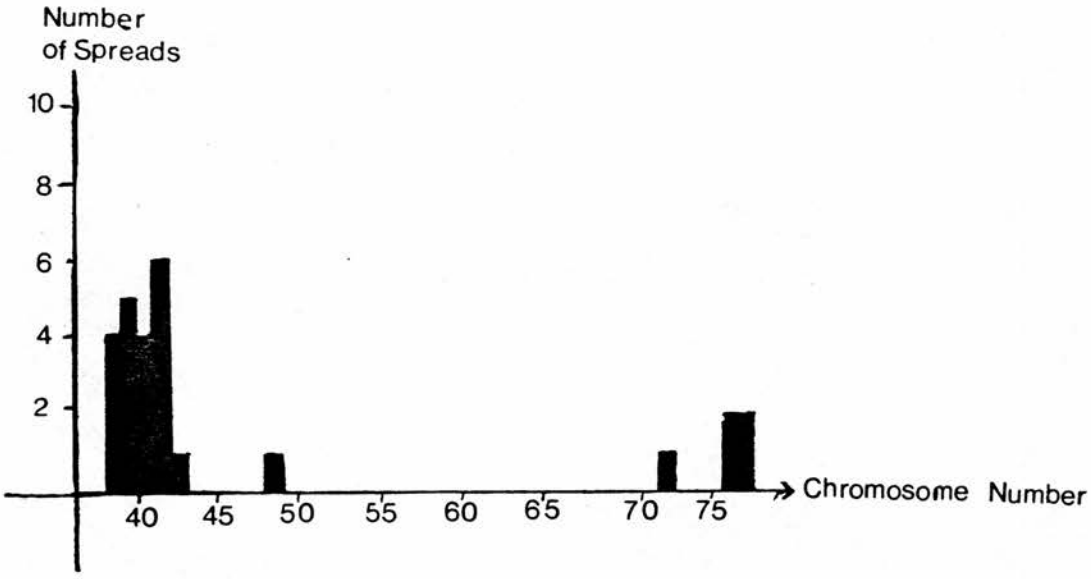

Number
of Spreads
10
8
6
4
2
40
45
50
55
60
65
70
75
Chromosome Number

Table 7:6

ASSAY OF MOD1,PYS AND PSA4 CELLS FOR THE PRESENCE OF PLASMINOGEN ACTIVATOR

a) Fibrin gel assay (1 experiment) shows the production of large quantities of plasminogen activator by Mod1 cells as measured by the extent of the lysis zone after samples of mediumincubated with these cells had been spotted onto the gel and left at 37°C for 1-2 hours.Medium conditioned with PYS cells also produced a measurable lysis zone within this time.medium conditioned by STO feeders (on which Mod1 cells were grown) or from PSA4 cells did not produce a lysis zone in this time.

b) Fibrin gel overlayer (4 experiments) shows the result of overlaying monolayer cultures of Mod1 cells (on feeders),PYS cells,PSA4 (on feeders) and STO feeders alone with medium containing thrombin,fibrin and with or without plasminogen.The results confirm those of 7:6a and show that Mod1 cells produce large amounts of plasminogen activator,PYS cells also produce this enzyme and the cell lines STO and PSA4 do not produce detectable amounts of PA within the 2 hour incubation period.

a)

Cell Line	Lysis Zone (mm)
Modl (+ sto feeders)	23
PYS	16
PSA4	0
STO feeders	0
Control	0

note: Control contained reaction mixture only and was not condtioned by incubation with a cell culture

b)

Cell Line	+ Plasminogen	- Plasminogen
Modl (+ sto feeders)	Complete Lysis	No Lysis
PYS	Approximately 50% Lysis	No Lysis
PSA4	No Lysis	No Lysis
STO feeders	No Lysis	No Lysis

note: results shown above are after 2 hours incubation at 37 C only and were consistent over four experiments

The results obtained with the fibrin gel overlayer assay,four experiments all together [Table 7:6b] confirm the production of large amounts of Plaminogen activator by Modl cells.Plates are completely lysed in approximately half the time required for lysis of the PYS plates.Therefore,using this assay,Modl plates were completely lysed in 1.5-2 hours and the PYS plates were at this time approximately 50% lysed.Control experiments showing that the STO feeder layer alone did not produce lysis of the fibrin gel in the presence of plasminogen were also consistent.

Because of the impossibility of obtaining confluent monolayer cultures of Modl cells,and also because of their slower growth rate compared to the PYS cells which become confluent within 2 days,the dishes containing the Modl cells had to be set up 1-2 days before the PYS cells to allow the densities of the two cell lines to be comparable,the PSA4 cells were also set up at the same time as the Modl cells.

It is known that STO fibroblasts are able to produce plasminogen activator.However the length of incubation time of these fibrin lysis experiments was very short (1-2 hours rather than 4) so the absence of detectable enzyme from STO cells in these experiments is likely to be due to the relatively low number of cells (10^6) present in feeder layers and the fact that they are not actively growing and so are not formed into colonies.Therefore the reduced time of incubation of the lysis experiments is probably insufficient to produce fibrin lysis in the STO dishes.Lysis zones tend to form above a growing colony (in PYS cells) or clump of cells (in the case of Mod1) indicating a local concentration of the enzyme.

The absence of lysis in dishes containing PSA4 cells confirm previous results that these ec cells do not produce PA when growing on STO feeders and reconfirm the feeder control result.

7.4.2. Alkaline Phosphatase

Modl and PSA4 cell lysates were assayed for the presence of alkaline phosphatase [Figure 7:6] ,and showed that the Modl cells produced none,or a very low level,of the enzyme,whereas the ec cells (PSA4) produced measurable amounts of the enzyme at quite low levels of cell protein,

Figure 7:6

PRODUCTION OF ALKALINE PHOSPHATASE BY MOD1 CELLS COMPARED TO THE EC LINE PSA4

a) Shows the result of plotting extinction (410nm) against µg of protein of enzyme mixtures containing cell lysates of either Mod1 or PSA4 cells.The units of alkaline phosphatase present in each sample was then calculated giving an average of;

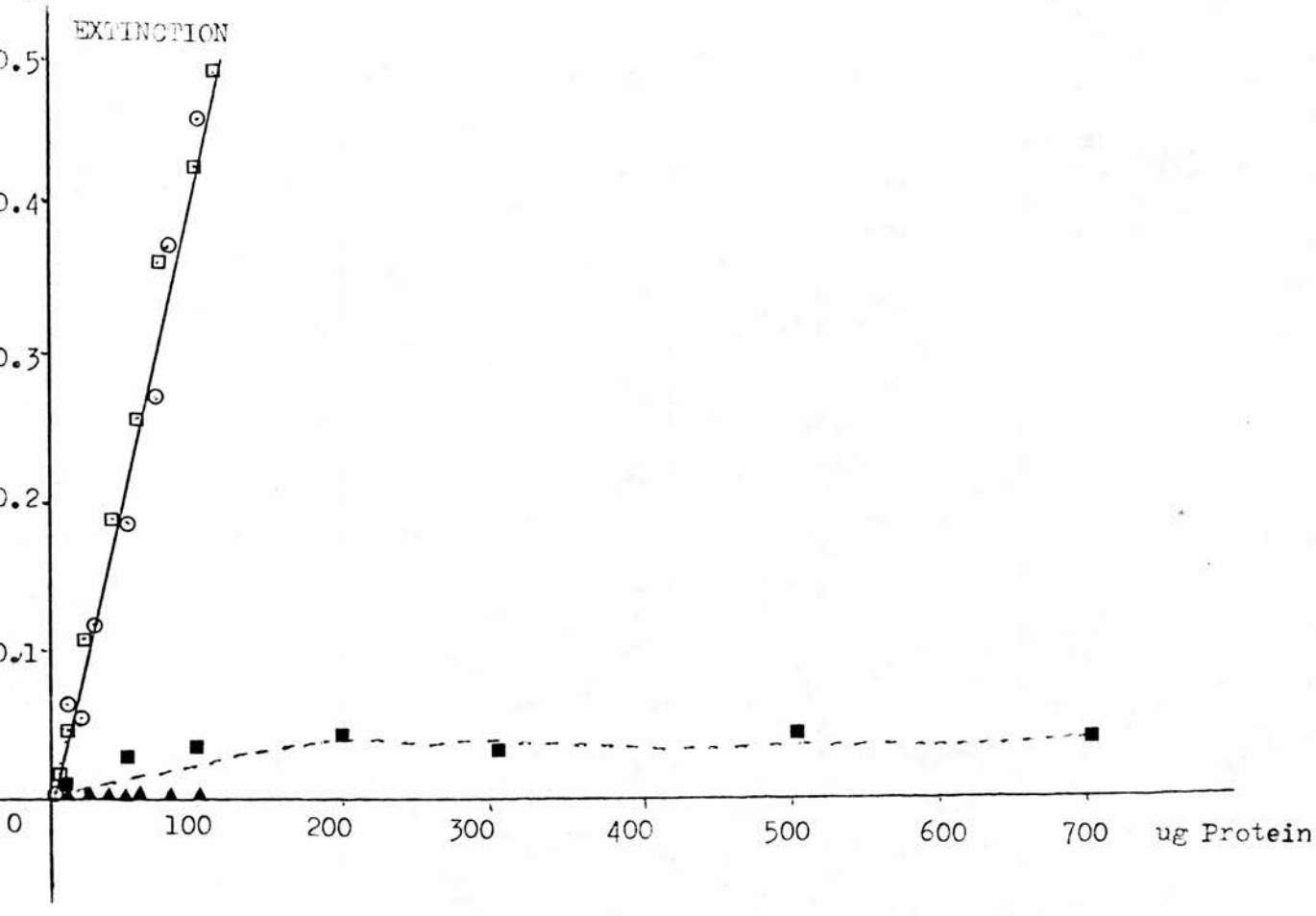
PSA4 : 3.42 units Alkaline Phosphatase per µg of protein
Mod1 : 0.09 units " " " " " "

Key:

○ - PSA4 (experiment 1)
□ - PSA4 (experiment 2)
▲ - Mod1 (experiment 1)
■ - Mod1 (experiment 2)

b) Shows the results of experiments 1 and 2 as plotted in 7:6a.

a)



b)

Modl

Extract (ul) 1	Extract (ul) 2	Protein (ug) 1	Protein (ug) 2	Extinction (410nm) 1	Extinction (410nm) 2	Units Alk.Phos. 1	Units Alk.Phos. 2	experiment
100	700	45	315	0	0.042	0	0.05	
80	500	36	225	0	0.04	0	0.068	
60	300	27	135	0	0.034	0	0.097	
40	200	18	90	0	0.044	0	0.188	
20	100	9	45	0	0.034	0	0.291	
10	50	4.5	22.5	0	0.028	0	0.479	
5	-	2.3	-	0	-	0	-	

PSA4

Extract (ul) 1	Extract (ul) 2	Protein (ug) 1	Protein (ug) 2	Extinction (410) 1	Extinction (410) 2	Units Alk.Phos. 1	Units Alk.Phos. 2	experiment
100	100	47	47	0.462	0.434	3.785	3.555	
80	70	37.6	32.9	0.374	0.274	3.83	3.206	
60	50	28.2	23.5	0.258	0.188	3.522	3.08	
40	30	18.8	14.1	0.19	0.12	3.891	3.277	
20	20	9.4	9.4	0.11	0.06	4.505	2.457	
10	10	4.7	4.7	0.046	0.07	3.277	5.734	
5	5	2.35	2.35	0.015	0.008	2.457	1.31	

that is 3.42 units of Enzyme per Img of protein (as measured by the biorad asssay see methods chapter [Figure 2:3]).At comparable levels of protein the assay on Modl cells yielded a result of 0 units of enzyme and it was only when concentrations of cellular protein 7-10 times that used in the PSA4 lysate assay were tested,that a low residual activity was detected in the Modl lysate.This activity was non-linear in relationship to the quantity of protein in the assay and was only just detectable (0.09 units per mg protein).The presence of large amounts of extracellular matrix in the protein preparations for this assay may interfere to some extent with the estimation of cell protein in the lysate and therefore may account for the non-linear relationship between the low level secretion and the estimated protein content.

7.4.3. L-Malic Enzyme

The presence or absence of this enzyme was initially tested for using monolayer cultures of the two cell lines PSA4 and Modl,and incubating the staining mixture on the growing cells for 20-30 minutes at 37°C. Unfortunately,because of the very different cell densities of the two cell lines in monolayer culture,the results were ambiguous and while the PSA4 cells appeared to stain positively it was not possible to say definitely whether or not the Modl cells had given a positive or negative result as the staining of individual cells was much more difficult to distinguish (R.L.Gardener pers.comm.).

7.4.4. Alphafoetal protein

Pre-fixed slides of both Modl and PSA4 cells were incubated with antibody to AFP coupled with HRP directed against the antibody.Results showed a low level of staining in the cytoplasm of the ec cell line (PSA4) and a variable level of staining in the Modl cell line which ranged from cells with very little staining to cells that were heavily stained,the latter group including a sub-population of cells which stained heavily in both the nucleus and the cytoplasm.Figure 7:7 illustrates these results.

7.5. Antigenic Properties of Mod1

Mod1 cells were tested [P.L.Stern,personal communication) for the presence of several cell surface markers by monoclonal antibodies which were known to react with markers on the H-2 histocompatibility complex and with antibodies raised against various ec cell surface markers.

7.5.1. H-2 complex markers

Neither of the monoclonal markers MCA 64 KbDb and MCA 67 Qa_2M were present on the surface of Mod1 cells.These monoclonals recognise the Db and the Qa_2 regions of the H-2 complex respectively.

7.5.2. Embryonal Carcinoma Cell markers

None of the ec cell surface markers tested were found to be present on the Mod1 cells.Antibodies tested were the forssmann antibody M1/22.25,5D4,SSEA1,SSEA111 and 2C5.

7.6. Inducement of Morphological change in Mod1 Cells

7.6.1. Off Feeder Layer Culture of Mod1 cells

Studies on the differentiation properties of Mod1 cells met with some initial difficulties since the cell line appeared to be completely dependant on the presence of an STO feeder layer for the survival of its cells.Later it was found that it was possible to obtain cell survival if the cells were plated out at high density using BFCS(20) or EC20 medium (appendix i).However it was observed that the morphology of the cells altered as they were removed from the feeder layer and this change was enhanced by growing them in medium which contained neither pyruvate,NEAAs nor B-mercaptoethanol.

Attempts to produce embryoid bodies from these cells resulted in the formation of irregular shaped bodies which appeared to contain varying amounts of extracellular material with small rounded cells associated with them [Figure 7:7].

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Figure 7:7

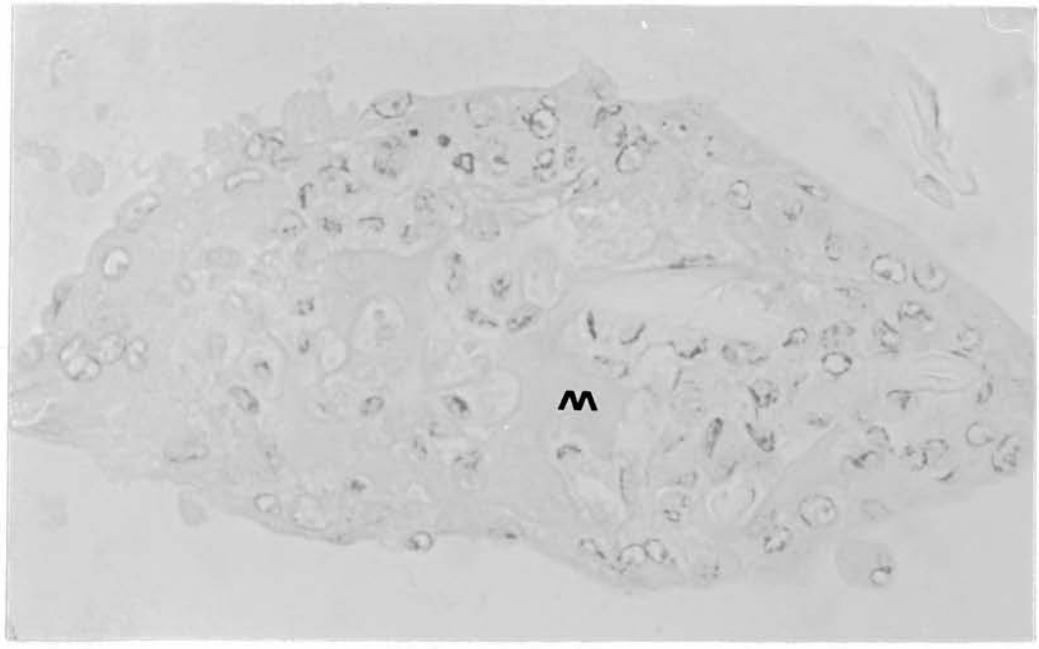
ILLUSTRATIONOF 'EMBRYOID BODIES' OBTAINED FROM MOD1 CELLS AFTER AGGREGATION IN PYRUVATE FREE MEDIUM (EC20)

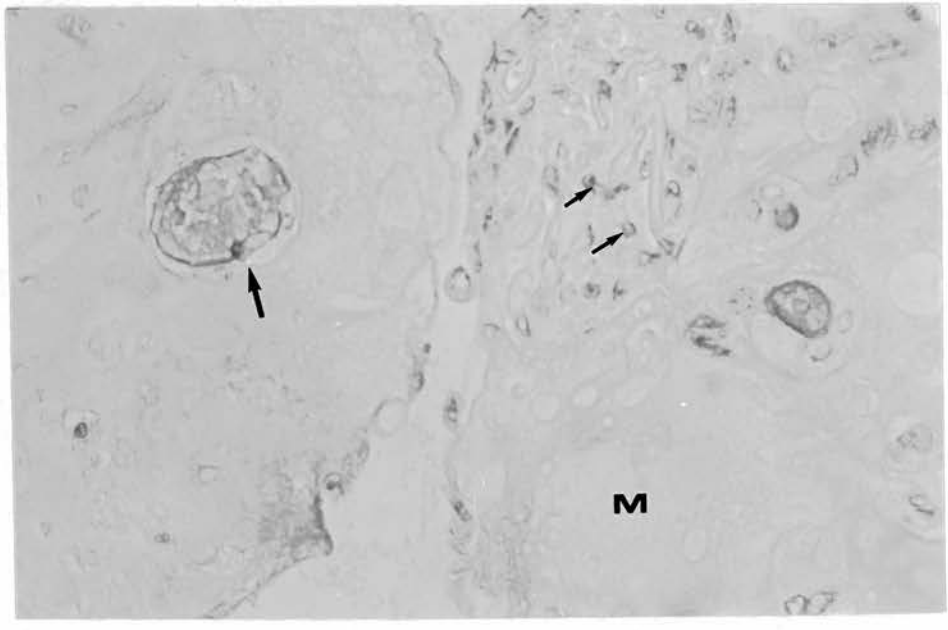
Illustrates the membrane bound structures obtained by culturing Mod1 cells in suspension aggregates.

A) Section of one such aggregate showing massive production of extracellular matrix (m) associated with small PAS negative cells,(magnification = 320).

B) Higher power (magnification = 1024) illustration of Mod1 aggregates illustrating an apparently organised circle of PAS positive staining cells (large arrow) which are secreting highly PAS positive material into a central lumen.Small arrows indicate the smaller PAS negative cells which form the majority of cells present under these conditions.

The sections in both A) and B) were stained with PAS (magenta positive) and counterstained with Haemotoxylin.


M


M

Some areas of these structures appeared to contain organised rings of cells secreting into a central lumen [Figure 7:8] but it is not known if these structres represent actual "differentiation".PAS staining of these aggregates has revealed that the extracellular matrix is PAS positive and stains bright magenta.While groups of cells staining very brightly with PAS were also observed the cells in general did not stain with PAS.

7.6.2. Morphological Changes

When removed from STO feeders Mod1 cells became less rounded up and more in contact with each other,they also appeared to be larger and multinucleate cells (2-6 nuclei per cell) were not uncommon.Secretion of extracellular matrix is much reduced under these conditions.Figure 7:9 illustrates the effect of plating Mod1 cells on gelatin in EC20 medium.Pictures were taken after 10 days incubation under these conditions.

7.6.3. Culture in Brl Medium

It has been recently demonstrated that it is possible to circumvent the morphological changes described in the previous section (6.6.2.) by the use of medium conditioned with Buffalo rat Liver cells [2....].The conditioned medium was used in a 60-75% "cocktail" with enriched (20% FCS) medium and B-mercaptoethanol was always added.When plated out onto gelatinised dishes in this medium,Mod1 cells grew normally,with light microscope (phase contrast) morphology resembling that of Mod1 cells growing on STO feeder cells [Figure 7:2] and producing an abundance of extraellular matrix.

7.6.4. Tumour Formation

In comparison to differentiating ec cell lines which produce differentiated tumours at the site of injection (and occasionally also secondaries in other organs) when injected into syngenic mice,no tumours were obtained in any of the C57Bl.Mod1^{null} male mice injected with Mod1 cells [2.4.6].The injected mice were observed for 12 months and then sacrificed and autopsied,but no trace of tumour material or other abnormalities were found.

Figure 7:8

MORPHOLOGY OF MOD1 CELLS WHEN CULTURED OFF STO FEEDER CELLS IN EC20

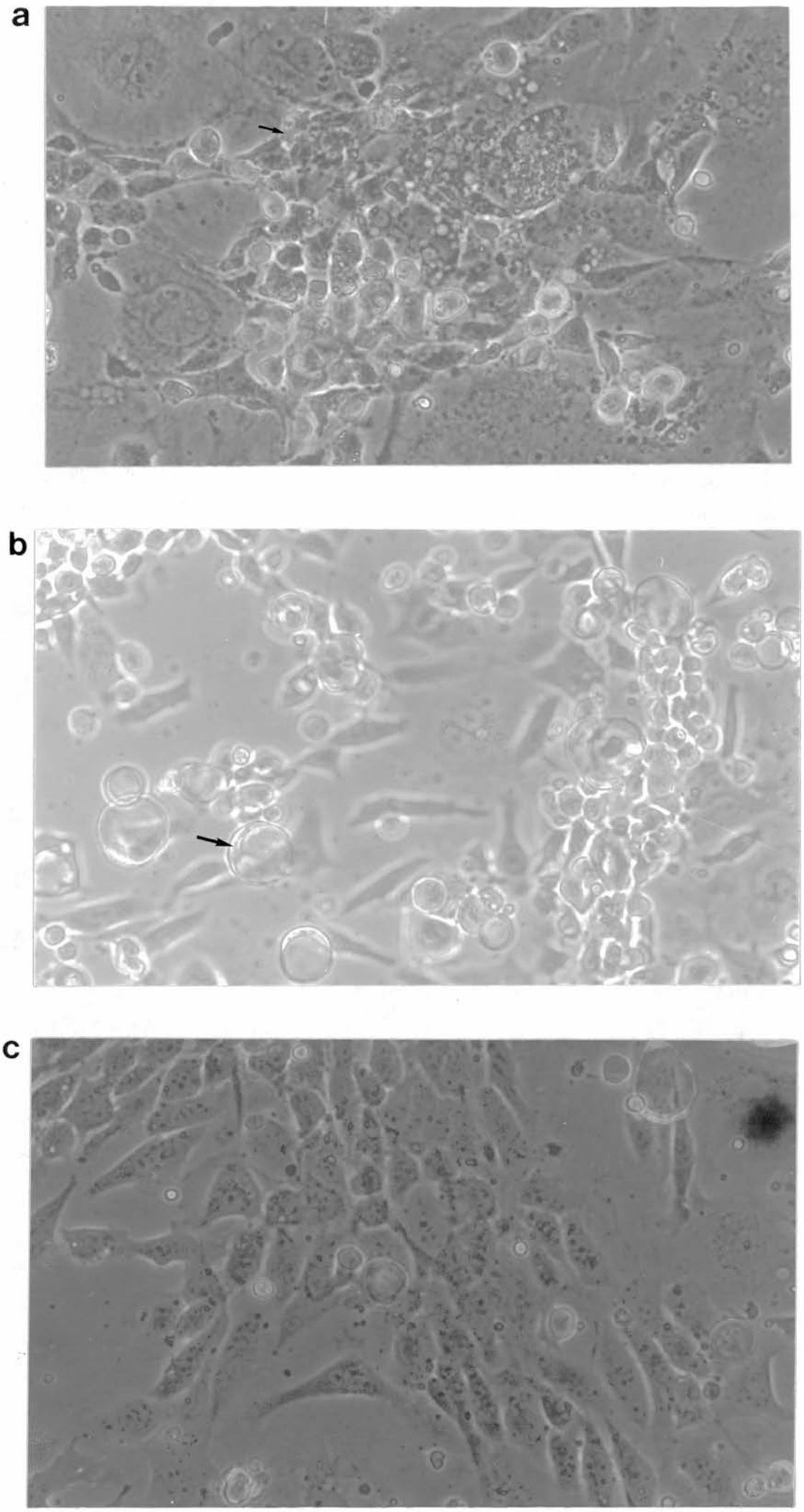
Shows the result of growing Mod1 cells at high density in gelatin treated tissue culture dishes in EC20 medium (containing 20% FCS and without pyruvate B-mercaptoethanol or non-essential amino acids).Pictures were taken after 10 days in these conditions.

a) Shows clumps of matrix producing cells which are apparently growing on top of more spread out and multinucleate cells (arrow).

b) Illustrates the formation of "endoderm vesicles" (arrow) apparently produced by small,non-contacting cells resembling those predominant in cultures of Mod1 cells growing on STO feeder layers (figure 2).

c) Shows an area of "triangular shaped" cells which are more spread out and form contacts with each other unlike cultures of Mod1 cells under normal conditions.

Magnification for a),b) and c) is 1280.


a
b
c

7.7. Summary and Discussion

This chapter has described the modification of methods for the isolation of cell lines from mouse blastocyst embryos.One of the lines isolated,Mod1,has been analysed in detail and has been cloned although no further work has been done on the clones of this line.Six other lines were also isolated and frozen down as permanent stock.

The method described resembles that of Axelrod for EK cell isolation [5,6] in that the most important aspects for the successful isolation of such cell lines were the use of minimal quantities of medium during the early stages,and the presence of STO feeder cells.It is postulated that the sucess of using small quantities of medium (10µl or less) is due to an autonomous conditioning mechanism exhibited by the cells upon which they are dependent for their proliferation and survival when in small numbers.This mechanism may become less important as the cell mass increases,or as the cells adapt to *in vitro* growth.

Removal of the outer trophectoderm layer of cells of the blastocyst embryos using Immunosurgery was successful and this method was used in the isolation of the line Mod1,the zona pellucida was removed using a standard enzyme method.Although subsequently lines were isolated from the whole embryo without the removal of either ZP or trophectoderm.

It is debateable which of the two methods is preferable,the use of immunosurgery ensures that the cell lines isolated are derived from ICM cells [55] but may interfere with these cells and cause them to behave abnormally.The use of pronase to remove the ZP has been shown to affect the cell-cell contact of ICM cells by alteration of cytokeratin and the myosin sheath [157] and also cell surface enzyme activity [146 and similar changes may also be incurred by the removal of the trophectoderm environment.

Further analysis of the line Mod1,isolated from the immunosurgically removed ICM of a C57BL.Mod1^{null} mouse blastocyst,has revealed that the line has a karyotype approximating diploid with a modal chromosome count of 41.As expected these cells do not appear to stain for the cytoplasmic L- Malic enzyme,which is absent from the cells of the C57Bl.Mod1^{null} mice but absolute confirmation of this was not possible due to the difficulty of comparing stained

monolayer preparations of Mod1 cells with those of 129 ec lines which were used as a control.The use of cell lines such as PYS may improve the results obtained from this method.

Morphologically Mod1 cells do not resemble cells of ec (or of EK) type although when initially isolated they did appear to have an ec morphology.They are not epithelioid and do not grow in the tightly packed colony formation characteristic of ec cells particularly when they are grown on STO feeder cells.Rather Mod1 cells are small,triangular shaped cells which tend to have a rounded-up appearance when grown in monolayer culture for several days and grow individually with little cell-cell contact.The growth of these cells seems to be associated with free-floating vesicles,resembling those found in association with endoderm cells.

However their morphology seems to alter with the culture conditions so that,if left in culture for several weeks or grown at high density in the absence of STO feeders they will become spread out and form cell-cell contacts.The most obvious characteristic of these cells is their production of large quantities of extracellular matrix when they are grown on STO feeder cells.When grown in EC20 medium in the absence of such feeder cells the production of this matrix almost ceases as the morphology of the cells alters.

It has been possible to form "aggregate structures" of Mod1 cells using a method similar to that used in making embryoid bodies from aggregates of EC cells.Under these conditions cells appear to grow around irregularly shaped masses of the extracellular matrix which is PAS positive staining.PAS stained paraffin wax preparations of sections of such structures show that the majority of cells do not stain with PAS but that matrix does.Small groups of cells associated with a strongly staining secretion also appear to stain with PAS.

These results suggest that Mod1 cells are of endoderm origin and that the PAS staining extracellular matrix is related to Reicherts membrane.The production of PAS positive extracellular material is characteristic of parietal endoderm cells which do not themselves stain positively for PAS.It has been shown [14] that visceral endoderm cells stain positively for PAS and it is likely therefore that Mod1 cells are composed either of primitive endoderm cells which are generating both types of endoderm or are predominantly composed of PE which under certain conditions is differentiating into VE.Evidence [1.3.4] of such

an interconversion between PE and VE,possibly with PrE as an intermediate stage,comes from studies on ec cell lines such as F9 which are capable of generating both types of endoderm depending on the conditions.

Peter Stern has obtained negative results with both monoclonal histocompatibility antibodies tested (MCA 64 KD-anti-H2,MCA 67 Qa2M-anti-Qa2) which is to be expected from embryo derived cells.In addition all of the monoclonal ec marker antibodies tested have produced negative results,(5D4,M1/22.25 [Forssman],SSEA1,SSEA111,2C5) he has concluded that this result is consistent with trophoblastic origin,but not confirmative.The absence of the Forssman antigen (M1/22.25) is an indication against both parietal and visceral endoderm on which it is usually expressed,and probably also PrE.The absence of either of the SSEA antigens rules out most origins except trophoblast and parietal endoderm.

With the aid of Dr J.Bell,slides of Mod1 cells have been stained immunohistochemically for the presence of AFP (alpha-fetoprotein),the results show positive but unevenly distributed staining of both cytoplasm and nuclei of Mod1 cells,compared with none (or very little) staining in controls.This result could indicate a variable production of AFP by either parietal endoderm or by trophoblast,neither of which in general synthesise AFP although both cell types may produce small quantities of it .Alternatively this result could indicate the presence of a mixture of Visceral/Parietal endoderm.

EM preparations of Mod1 reveal cells with a well developed rough ER and Golgi body system which are obviously actively secreting.In addtion areas of microvilli have been found.These features are mostly consistent with a PrE or PE origin and compare closely with em pictures of PE and PrE taken from differentiated embryoid bodies [14].Some of the features,for example the presence of electron lucent vacuoles,and areas of high density microvilli are,however more consistent with early VE morphology and support the "Mixed Cell" postulate.

Most of the features described are consistent with a PE or "Mixed Cell" (PE,VE,PrE) origin for Mod1,although the absence of cell surface markers and the presence of AFP are more consistent with trophoblast.Reicherts membrane is situated between these two cell type and it is therefore possible that it is contributed to by both Trophoblast and PE cells.This question could be further

clarified by analysis of Mod1 cell lysates for the presence of the hormone enzyme B-hydroxy steroid dehydrogenase (BHSD).Preliminary investigations,using chromatography of radioactive substrates,has shown good separation and identification of the substrate and end-product,but as yet no clear result has been obtained as to whether or not the Mod1 cell line produces the B-HSD enzyme.

In summary therefore further investigation of the properties of Mod1 cells confirm that this cell line is unlikely to be of ec origin and mostly indicate that these cells are composed of primitive endoderm which is generating both PE and VE in varying quantites with PE predominating,although cell surface marker experiments indicate that these cells may be of extraembryonic origin.

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APPENDIX i

Culture Media and Saline Solutions

i)All Culture media were based on the standard recipe for complete medium (CM) which was made in either 100ml or 500ml quantities and consisted of the following;

	Amount per 100ml medium	Final Concentration
Glasgows Modified	9mls	1x
Eagles Medium (10x)		
Non-Essential Amino Acids (NEAA's)	1ml	1x
Sodium Pyruvate(Pyr)	1ml	1mM
L-Glutamine (Gln)	1ml	1mM
B-Mercaptoethanol	0.1ml	0.1mM
Sodium Bicarbonate	3.3ml	
Deionised Distilled Sterile Water (DDW)	75ml	-

for GMEM see:Stoker,M.G.P. & Macpherson,I. Virology 14 359-370.

NEAA contains O.1mM quantities of each of the following amino acids,L-Alanine,L-Asparagine H O,L-AsparticAcid,Glycine,L-Glutamic Acid and 0.2mM quantities of L-Proline and L-Serine.
In addition to the above complete medium was also supplemented with calf serum 10-20% (either new born calf serum (NCS),foetal calf serum (FCS) or nu-serum as indicated in the text and below).
Variations of complete medium are as follows;

CM(FCS) - 10% Foetal calf serum
CM(NCS) - 10% New born calf serum
CM(20) - 20% Foetal calf serum
CM(10) - 10% calf serum (as indicated)
CM(X) - serumless medium with calf serum replaced with 10mls PBS per 100mls medium to retain the same concentrations of other ingredients.

ii) Embryoid body culture medium (used for suspension aggregates) was complete medium without Pyr & NEAA's supplements;

EC 10 - with 10% calf serum (usually NCS but ocassionally FCS as indicated in the text)
EC 20 - with 20% FCS

as indicated in the text)
EC 20 - with 20% FCS

iii) PBSA is a standard saline solution (Flow,see methods 'Materials' section).

PBS is PBSA supplemented with $CaCl_2.2H_20$ (57mg/400ml PBSA) and $MgCl_2.6H_2O$ (40mg/400ml PBSA).

DECLARATION

I declare that the work reported by me in this thesis is,with the exceptions mentioned,entirely my own work and was all performed by myself.

Janet Smith